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Passive Cutaneous Anaphylaxis with Antigen-Antibody Complexes and Additional Antigen.* (24073)

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The effect of antigen-antibody precipitates on tissues was described as a type of foreign body reaction by Opie(1). Recent investigations have demonstrated a variety of inflammatory and "immune" reactions associated with administration of antigen-antibody complexes(2-5). The continued potential for such combined antibody to react *in vivo* with additional antigen has not been demonstrated. In the present study the ability of antibody in washed specific precipitate to sensitize tissues to a subsequent injection of antigen is demonstrated. Mixtures of antigen with antibody ranging from great antigen excess to antibody excess, as described below, were also effective. The amounts of combined antibody which sensitize approximate the minimum amounts required for sensitization by uncombined antibody.

Methods. Guinea pig antisera to 4 times recrystallized egg albumin were analyzed for antibody nitrogen by the Markham modification of the micro-Kjeldahl procedure(6). Appropriate dilutions were made of serum stored at 4°C and lacking complement activity. To aliquots of the antibody solutions varying amounts of antigen were added, as indicated in the Table. Since these solutions were made to contain minute quantities of antibody, precipitation was not ordinarily visible. The presence of antigen excess was confirmed by capillary precipitin test. Specific precipitate was prepared in quadruplicate by adding a slight excess of egg albumin to a pool of guinea pig antisera containing 146 μg AbN/ml. The precipitates were washed 3 times in cold saline, and duplicate precipitates were analyzed for nitrogen content. Tests of the first supernate confirmed the presence of excess antigen. For injection, aliquots of precipitate were vigorously agitated and suspended in saline. Albino male guinea pigs weighing 250 $\pm$ 50 g were injected intracutaneously in the flank with 0.1 ml of antigen-antibody solution, a suspension of washed specific precipitate or other materials to be tested. Animals were challenged to determine specific reactivity of

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TABLE I. Passive Cutaneous Anaphylactic Reactions in Guinea Pig Skin with 0.1 ml of Antigen-Antibody Complexes Followed by Antigen Intravenously.

	Ratio AbN/EaN at skin site								
	Antigen excess					"Equivalence"	Antibody excess		
μg AbN at skin site	1/60	1/43	1/6–1/4	1.66	4	± 7	30	150	No antigen
.2						+++ ++			
.15	+++ +++	++++ ++ +	++++ +++ ++	++++ +++	++++ +++		++++ +++ +++	++++ +++ +++	++++ ++++
.075		0							++++
.03		+ + 0	++++ ++++ 0						++++
.02						++			
.01		0 0	+++ ++ +	++ + 0					+++ ++ ++ +

Reactions in the "equivalence" zone were induced with thrice washed specific precipitates prepared in slight antigen excess, followed, as in all other instances, by the inj. of antigen intrav.

the injected skin sites for passive cutaneous anaphylaxis(7) at either 2 or 18 hours after intracutaneous injection. One mg egg albumin in 1 ml saline and 0.5 ml of a 0.5% solution of T-1824 (Evans Blue Dye) were injected together intravenously. With the amounts of antibody used, reactions were generally more marked at 18 hours, and most of the results were obtained using this interval between sensitization and challenge. Furthermore, non-specific dye leakage due to trauma or other factors does not occur at this interval. Skin sites were examined for extravasation of dye 15 minutes after challenge. The area of dye leakage was measured after the animals were sacrificed, bled out and the skin reflected. Reactions were graded one to 4 plus, according to the size and intensity of the blue spot as previously indicated(8).

Results. Intracutaneous injection of 0.1 ml of a suspension of washed specific precipitate containing 0.2 μg AbN sensitized the skin of 2 guinea pigs so that intravenous injection of 1 mg egg albumin and blue dye after 18 hours elicited distinct reactions of passive cutaneous anaphylaxis. When as little as 0.02 μg AbN was present in the sensitizing precipitate, a positive reaction was also elicited. The injection of dye alone produced no dye localization at the site of injection of the precipitate. Varying quantities and proportions of antibody mixed with antigen were injected intracutaneously, one site in each guinea pig. The results of subsequent challenge with intravenous antigen and dye are indicated in the Table. With 0.15 μg AbN and excess antigen, 2 skin sites showed erythematous reactions 18 hours after sensitization and prior to challenge. Following challenge, these sites gave passive cutaneous anaphylactic reactions. Comparable sites in control animals did not cause dye localization when dye alone or antibody and dye were injected 2 or 18 hours after sensitization. A skin irritating effect has been noted for antigen-antibody mixtures in larger amounts than those used here(4).

In the Table it is apparent that antigen-antibody complexes in the range of antigen excess sensitize the guinea pig tissue to passive cutaneous anaphylaxis. This occurred regularly with 0.15 μg AbN in the injected mixtures containing approximately 400 times the amount of antigen present at "equivalence." In a few instances, it was found that 0.03 μg AbN and 0.01 μg AbN were also capable of sensitization. Complete titrations of excess antigen were not carried out at these levels of antibody nitrogen. In the region of moderate antigen excess the reactions elicited by subsequent antigen were comparable to

those caused by complexes in antibody excess, and those with antibody alone as the sensitizing agent.

A preliminary test with a polysaccharide antigen (Streptococcus C) and rabbit antibody indicates that the phenomenon of sensitization by antigen-antibody complexes may be accomplished with this system as well.

Discussion. The specific combination of antigen with antibody has been described by physico-chemical laws(6). The results of this combination are evident *in vitro* in the precipitin test and its derivative reactions, and *in vivo* in specific immune or allergic phenomena(6). In the present study the ability of specific precipitates and of antigen-antibody mixtures in great antigen excess to sensitize tissues to subsequent anaphylaxis suggests that optimal biological reactivity of antibody persists, despite apparent neutralization of antibody in *in vitro* tests. As little as 0.01 and 0.03 μg AbN, amounts approximating that required for threshold sensitivity, remain capable of sensitization despite the initial presence of excess amounts of antigen. When amounts of this order are utilized, it is improbable that multiple antigen-antibody systems account for the biological reactivity noted. In addition, the skin irritating activity of the complex(4) is considerably diminished so that control injections with dye alone do not result in dye localization. Apparently the antibody continues to react with antigen until extreme antigen excess is present, an indication of greater combining ratios of antigen with antibody than is ordinarily appreciated from *in vitro* tests.

Alternatively, the dissociation *in vivo* of antigen from antibody with conservation of the reactive properties of the antibody may be postulated. The small amount of antibody used suggests that antibody may be reutilized almost quantitatively in some processes of immunity and allergy. The present data do not permit the exclusion of either alternative.

Summary. Small amounts of antibody and excess antigen sensitize guinea pig skin so that challenge with intravenous antigen and dye elicits passive cutaneous anaphylaxis. The significance of the biological reactivity of antigen-antibody complexes is briefly discussed.

1. Opie, E. L., *J. Immunol.*, 1924, v9, 259.
2. Germuth, F. G., Jr., McKinnon, G. E., *Bull. Johns Hopkins Hosp.*, 1957, v100, 187.
3. Trapani, I. L., Garvey, J. S., Campbell, D. H., *Science*, 1958, v127, 700.
4. Ishizaka, K., Campbell, D. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 635.
5. Cochran, C. G., Weigle, W. O., *Fed. Proc.*, 1958, v17, 507.
6. Kabat, E. A., Mayer, M., *Experimental Immunochemistry*, Charles C. Thomas, Springfield, 1948.
7. Ovary, Z., *Trans. VI Int. Congress Microbiol.*, Rome, 1953.
8. Rosenberg, L. T., Chandler, M. H., Gordon, A. S., Fischel, E. E., *J. Immunol.*, in press, 1958.

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Sendai Virus Antibody in Acute Respiratory Infections and Infectious Mononucleosis. (24074)

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Japanese investigators have recently studied a virus, variously referred to as newborn pneumonitis virus, hemagglutinating virus of mice, hemagglutinating virus of Japan, and Sendai virus, isolated from cases of human respiratory infection(1), from laboratory mice(2), and swine(3). First reports of virus isolations and of serologic evidence indicating human infection with this virus were limited to Ja-

* Data were submitted to Graduate School, University of Wisconsin, in partial fulfillment of requirements for degree Doctor of Philosophy.

pan, but recently Russian workers(4,5) reported isolation of the agent in Vladivostok from 5 patients with influenza-like illnesses. In 1955 Jensen *et al.*(6) presented serologic evidence suggesting that the population of United States has had contact with this or an antigenically closely related agent, but with the finding that infection with mumps virus results, in appearance of antibody reactive with Sendai virus†(7,8) the question remains as to whether Sendai virus itself is a cause of inapparent or overt human disease in the United States. In studies on respiratory infection in University of Wisconsin students a number of patients were found to develop serum reactivity with Sendai virus during illnesses that did not appear to be mumps infections. Our study of these illnesses is reported here.

Methods. The Sendai Strain of hemagglutinating virus of Japan, here referred to as Sendai virus, and Enders strain of mumps virus were used and cultivated in the allantoic sac of chicken embryos using standard methods previously described(7). *Serologic technics.* Hemagglutination and hemagglutination-inhibition tests were performed as outlined by Committee on Standard Serologic Procedures in Influenza Studies(9). Sera were treated with receptor-destroying-enzyme as previously described(7). Four hemagglutinating units of virus were used in hemagglutination-inhibition tests and titers are expressed as reciprocal of initial serum dilution. Complement-fixation tests were performed using allantoic fluid antigen and 2 full units of complement. Neutralization tests *in ovo* employed approximately 200 egg-infectivity doses of virus and serial dilutions of serum previously inactivated at 56°C for 30 minutes. Virus-serum mixtures were incubated 1 hour at 4°C and inoculated intra-allantoically. Inoculations were made into 11-day-old embryos, and incubated an additional 2 days before allantoic fluids were tested for presence of hemagglutinins. The 50% infectivity end points were calculated by the method of Reed and Muench(10). *Virus isolation procedures.* Penicillin and streptomycin were added to throat washings in concentrations of 1000 units and 1 mg/ml respectively. Six 13-day-old embryonated eggs were inoculated intra-amniotically with 0.2 ml of undiluted throat-washing and 6 three-week-old mice were inoculated intranasally with 0.05 ml while under light ether anesthesia. Embryonated eggs were incubated for 3 or 4 days at 35°C and amniotic fluids were then tested for presence of hemagglutinins using both chicken and guinea pig erythrocytes in 0.5% suspensions. At least 2 serial passages were made in eggs before specimens were considered negative. Mice were observed daily for 7 days and then sacrificed. Lungs were collected and emulsified in a glass tissue grinder to make a 10% suspension which was lightly centrifuged. Antibiotics were added to supernatant fluid and it was again inoculated intranasally into mice. Three serial passages in mice were made with each specimen.

Results. In view of reports by Japanese workers associating Sendai virus infection with influenza-like respiratory infections and pneumonitis, it seemed possible that this virus might play some part in the cause of non-bacterial, respiratory infections found in university student population. Paired sera were available from 85 cases variously diagnosed clinically as "grippe," bronchitis, or "influenza" which could be grouped together as non-bacterial respiratory infections and were generally characterized by fever, generalized muscular aches, headache, occasional sore throat or cough, paucity of abnormal findings on physical examination, and failure to develop a neutrophilia. There were also 50 cases that had been diagnosed as viral pneumonia or primary atypical pneumonia because of evidence of pneumonitis on physical examination or chest roentgenogram in addition to usual signs and symptoms of non-bacterial respiratory infection. Acute and convalescent serums from these 2 groups of patients were studied for antibody rises to Sendai virus.

† The designation "Sendai virus" is not one favored by Japanese workers and is not that previously used by us, but it has an advantage of succinctness and for that reason will be used here.

TABLE I. Antibody Titers against Various Antigens in Cases Diagnosed as "Grippe" and Primary Atypical Pneumonia and Developing Significant Antibody Rises to Sendai Virus.

Case No.	Antibody titers against: Sendai virus H.I. Acute serum	Sendai virus H.I. Conv. serum	Sendai virus Neut. *in ovo* Acute serum	Sendai virus Neut. *in ovo* Conv. serum	Mumps virus H.I. Acute serum	Mumps virus H.I. Conv. serum	Mumps virus C.F. Acute serum	Mumps virus C.F. Conv. serum	Heterophile antibody	Cold agglutinins	Streptococcus MG agglutinins Acute serum	Streptococcus MG agglutinins Conv. serum
1*	8	32	28	128	32	32	4	4	<20			
2	8	32	11	48	<8	<8	<2	<2	<20	<8	<8	
3	8	32	<4	32	"	16	4	4	"	"	"	
4	<8	16	12	16	"	<8	<2	<2	<20			
5	"	64	8	8	"	"	4	16	"			
6*	"	32			"	"	4	4	"	64		
7	16	128	<16	32	"	"	<2	<2		8	2	2
8	8	32			"	"	"	"			2	4
9	<8	16	12	23	"	"	16	16	"	32	4	4
10	"	16	8	>32	"	"	8	8		<8	4	32
11	16	64	<8	<8	"	"	8	8		"	4	8

* Cases 1 to 5 diagnosed as "grippe" and Cases 6 to 11 as primary atypical pneumonia. † For heterophile antibody and cold agglutinins 2 numbers indicate 2 or more serum specimens tested but not that they were acute and convalescent specimens.

Of 85 non-bacterial respiratory infections, 5 were found (Table I, Cases 1 to 5) to have 4-fold or greater increases in hemagglutination-inhibiting antibody against Sendai virus and 3 of these also had significant increase in neutralizing antibody during the illness. Serums of these cases had previously been tested and found negative for increases in hemagglutination-inhibiting antibody against influenza A, B, and C. Two patients had 4-fold increases in either hemagglutination-inhibiting or complement-fixing antibody against mumps virus, although they were not suspected of having mumps during their clinical illnesses.

Of 50 cases labelled viral pneumonia or primary atypical pneumonia, 6 (Table I, Cases 6 to 11) had 4-fold or greater increases in antibody against Sendai virus measured by hemagglutination-inhibition. When 4 of these were tested by neutralization *in ovo,* 2 had significant increases in neutralizing antibody. In none of the 6 cases was there any increase in hemagglutination-inhibiting or complement-fixing antibody against mumps virus. Sera from these patients had previously been studied by complement-fixation test for presence of antibodies against psittacosis, Q-fever, and adenoviruses and no serologic evidence of infection with these agents had been found. Five patients had definite roentgenographic evidence of pneumonitis and diagnosis of primary atypical pneumonia for the sixth patient (Case 9) was based on indication of pneumonitis on repeated physical examination and a cold agglutinin titer of 1:32. During hospital stay of 5 of the 6 patients, serum was tested for presence of cold-agglutinins on one or more occasions (Table I). Two of these had titers of 1:32 and 1:64 respectively. Repetition of cold agglutinin titrations at time of measurement of antibody against Sendai virus was not done because of limited supplies of serum and serious question that the paired serums had been properly collected for cold-agglutinin measurements. Measurements of Streptococcus MG agglutinins in acute and convalescent serums were made, however, and it was found (Table I) that in serum of 1 patient agglutinins appeared during the illness.

TABLE II. Antibody Titers and Physical Signs in Cases Diagnosed as Infectious Mononucleosis and Developing Significant Antibody Rises to Sendai Virus.

Case No.	Antibody titers to: Sendai virus H.I. Acute serum	H.I. Conv. serum	Neut. *in ovo* Acute serum	Neut. *in ovo* Conv. serum	Mumps virus H.I. Acute serum	H.I. Conv. serum	C.F. Acute serum	C.F. Conv. serum	Heterophile antibody	Physical signs: Exudative pharyngitis	Lymphadenopathy	Hematology—>50% lymphocytes	Abnormal liver function	Hepatomegaly	Splenomegaly
12	64	>256	213	1296	32	32	2	2	1024	+	+	+			+
13	<8	32	<4	4	<8	16	<2	<2	64 >128	+	+	+	+	-	+
14	64	>256	16	78	32	32	4	16	128	+	+	+		-	+
15	8	32	<6	24	16	,,	<2	<2	256	+	+	+	+	+	+
16	<8	32			<8	<8	,,	,,	64	-					
17	32	128			32	32	2	8	128 256	-	+	+	+	+	+
18	64	256			,,	,,	8	8	<20	-				+	+
19	8	32			<16	<16			,,	-		-			
20	<8	16			<8	<8	<2	<2	128	+	+	+			
21	16	64			,,	,,	,,	,,	<20	+			+		
22	<8	16			,,	,,	,,	2	64	+		+			
23	,,	64			,,	,,	8	8	256	+	+	+		+	+
24	,,	128			,,	,,	4	4	128	+	+	+		+	+

Both influenza-like illnesses and pneumonia groups occurred as sporadic cases during winter months for 2 years and were not associated with sharp outbreaks of infection.

Those patients who developed antibody reactive with Sendai virus frequently had moderate, generalized lymphadenopathy and sometimes had moderate lymphocytosis. In 1 patient lymphocytes were reported as "atypical" and in another heterophile antibody appeared in serum during the illness. This led to study of patients presenting syndrome of infectious mononucleosis. Cases that did not exhibit all usual features of infectious mononucleosis were initially selected for study in the belief that illnesses associated with Sendai virus might more likely be found among poorly defined illnesses resembling infectious mononucleosis in certain respects, but lacking a heterophile antibody response. Continued study, however, revealed cases in which all features usually identified with infectious mononucleosis were clearly present and in which there was a definite increase in antibody reactive with Sendai virus during the illness.

Paired serums from 110 patients hospitalized during 1954 and 1955 with diagnosis of infectious mononucleosis were examined. Thirteen patients in 2 years (Table II) had 4-fold or greater rises in hemagglutination-inhibiting antibody against Sendai virus. Titration of 4 of these by neutralization *in ovo* demonstrated (Table II) that increase in hemagglutination-inhibiting antibody was accompanied by increase in neutralizing titer. Two of the 13 patients also developed low levels of hemagglutination-inhibiting or complement-fixing antibody against mumps virus. Measurement of hemagglutination-inhibiting antibodies against Newcastle disease virus, against a 1954 strain of influenza A virus, a 1954 strain of influenza B virus, and influenza C virus were carried out with 9 pairs of serums and no increases in antibody reactive with these viruses could be detected.

Many of these cases had the cardinal features of infectious mononucleosis: exudative pharyngitis, lymphadenopathy, lymphocytosis with atypical cells, and heterophile antibody

in the serum (Table II). Several had splenomegaly and some had evidence of liver involvement.

Since the question of whether Sendai virus was the agent causing the 24 illnesses exhibiting anti-Sendai antibody rises would be considerably clarified by isolation of virus from these patients, efforts were made to recover a virus from throat washings. In connection with other studies throat washings from 7 patients previously had been used to inoculate cultures of HeLa cells and no isolation of a viral agent had been accomplished. During present study, throat washings were available from 17 of the 24 patients but these specimens had been stored at –20°C for 6 months to 2 years. Although this storage markedly diminished the likelihood of a successful isolation of Sendai virus, attempts at isolation of the virus were, nevertheless, made. Two successive passages in amniotic sac of chicken embryos and 3 serial passages in Swiss mice by intranasal route for each specimen failed, however, to yield evidence of a viral agent culturable by these methods.

During these studies paired serums from febrile illnesses of known etiology were examined on the possibility that the factor in serum reactive with Sendai virus might be a non-specific one arising in association with a febrile episode. Available paired serums from cases in which the possibility of infection with Sendai virus or mumps virus could clearly be excluded were few in number, but 41 cases consisting of patients with streptococcal pharyngitis (confirmed by culture), scarlet fever, cellulitis, poliomyelitis, varicella, sinusitis, and measles were examined and an increase in reactivity with Sendai virus failed to appear during these illnesses.

Discussion. The factor in serum reactive with Sendai virus appeared to be antibody since it was stable at 56° C for ½ hour, was not destroyed by crude *V. comma* culture filtrate, showed increase in titer during illness, and was demonstrable by both neutralization and hemagglutination-inhibition technics. In most cases there was no evidence, either clinical or serologic, that increase in antibody reactive with Sendai virus could be attributed to infection with mumps virus. Four patients, however, did have small increases in antibody reactive with mumps virus, although there was nothing about their clinical syndromes to suggest that they had mumps infections. It can be said that these latter patients may have been infected with mumps virus and therefore produced antibody reactive with Sendai virus, or it may be that they were infected with Sendai virus or an agent antigenically related to both mumps and Sendai viruses, and hence could have responded with antibody reactive with these 2 viruses. Our cases with influenza-like illness and pneumonia were similar in many respects to cases in Japan, Great Britain(8,11,12), and Russia(5) believed to be caused by Sendai virus. It is noteworthy that of 2 illnesses with antibody increases to Sendai virus previously reported from the United States(4), one had an influenza-like illness and one atypical pneumonia. A good possibility, however, is that these infections may have been due to another viral agent antigenically related to Sendai virus. The fact that infection with mumps virus may result in sharp increases in antibody reactive with Sendai virus and the recent isolation by Chanock *et al.*(13) of a myxovirus that stimulates production of antibodies reactive with Sendai virus serve to emphasize this possibility which seems particularly plausible in those cases with typical syndrome of infectious mononucleosis. Patients developing antibody against Sendai virus represented only 13 of 110 examined and were indistinguishable in other respects from other cases of infectious mononucleosis. The etiologic agent of infectious mononucleosis (at present unidentified), or at least some strains of it, may be antigenically related to Sendai virus and the right circumstances, or infection with certain strains could result in development of cross-reacting antibody. A factor is known to appear in serum of some patients with infectious mononucleosis that agglutinates erythrocytes previously treated with Newcastle disease virus (14, 15), and although the significance of this reaction is not yet clear, the fact that the reaction is concerned with another myxovirus lends some additional support to the possibility that a virus of this group, perhaps antigenically related to both Sendai virus and

Newcastle disease virus, may be involved in the etiology of infectious mononucleosis. A further suggestion that the serologic reactions reported here are not explained by Sendai virus infection *per se* lies in the fact that many studies have been carried out in this country in which attempts at isolation of virus from patients with non-bacterial respiratory infections, viral pneumonitis, and infectious mononucleosis have employed chicken embryos and mice in a manner that would be expected to result in isolation of Sendai virus if it were present. Sendai virus is also cultivated quite readily in several lines of tissue culture cells (16) frequently used in such studies in recent years. Failure to isolate this virus suggests that one, or perhaps several, much more fastidious agents may be responsible for the observed antigenic stimulation.

Summary. Measurement of antibodies in acute and convalescent serums from college students hospitalized because of acute, non-bacterial respiratory infections revealed 5 cases of influenza-like illness and 6 cases of viral pneumonia with 4-fold or greater increases in reactivity with Sendai virus measurable by hemagglutination-inhibition and neutralization *in ovo*. Thirteen of 110 patients hospitalized with the diagnosis of infectious mononucleosis were found to develop increases in serum reactivity with Sendai virus during their illnesses. Efforts to isolate a virus from throat washings of 17 of these patients were unsuccessful. The possible role of Sendai virus in the etiology of these illnesses and the significance of the antibody responses were discussed.

1. Kuroya, M., Ishida, N., *Yokohama M. Bull.,* 1953, v4, 217.

2. Fukumi, H., Nishikawa, F., Kitayama, T., *Jap. J. M. Sc. and Biol.,* 1954, v7, 345.

3. Sasahara, J., Hayashi, S., Kumagai, T., Uamamoto, Y., Hirasawa, N., Munekata, K., Okaniwa, A., Kato, K., *Virus,* 1954, v4, 131.

4. Gerngross, O. G., *Problems in Virology,* 1957, v2, 71.

5. Gorbunova, A. S., Gerngross, O. G., Gnorizova, V. M., Bukrinskaia, A. G., *ibid.,* 1957, v2, 76.

6. Jensen, K. E., Minuse, E., Ackermann, W. W., *J. Immunol.,* 1955, v75, 71.

7. DeMeio, J. W., Walker, D. L., *J. Immunol.,* 1957, v78, 465.

8. Gardner, P. S., *Brit. M. J.,* 1957, v1, 1143.

9. Committee on Standard Serological Procedures in Influenza Studies, *J. Immunol.,* 1950, v65, 347.

10. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

11. White, G. B. B., Gardner, P. S., *Brit. M. J.,* 1957, v1, 381.

12. Sommerville, R. G., Carson, H. G., *ibid.,* 1957, v1, 1145.

13. Chanock, R. M., Parrot, R., Cook, K., Andrews, B., Bell, J., Reichelderfer, T., Kapikian, A., Mastrota, F., Huebner, R. J., *New England J. Med.,* 1958, v258, 207.

14. Burnet, F. M., Anderson, S. G., *Brit. J. Exp. Path.,* 1946, v27, 236.

15. Evans, A. S., Curnen, E. C., *J. Immunol.,* 1948, v58, 323.

16. Parker, H., Walker, D. L., unpublished observations.

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Influence of Various Agents on Mast Cells Isolated from Rat Peritoneal Fluid.* (24075)

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There is considerable evidence that mast cells contain and are able to release acid mucopolysaccharides, histamine, and, in certain species, serotonin(1). Mast cell degranulation with loss of metachromatic material is effected by histamine-liberator substances *in*

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vivo(2). However, Riley *et al.*(3) were not able to demonstrate conformity between liberation of histamine and heparin in such experiments. In cheek pouches of living hamsters Asboe-Hansen and Wegelius(4,5) observed release of mast cell granules not only in response to histamine liberators, but to histamine itself, serotonin, certain physiological fluids, hyaluronidase, and experimentally induced edema. They advanced the hypothesis that degranulation may be a physiological response to edema, and that acid mucopolysaccharide from mast cells binds extracellular water to form mucinous ground substance. The present investigation was designed to supplement such *in vivo* studies by observations on the *in vitro* influence of these agents, along with others, on mast cells isolated from rat peritoneal fluid.

Methods. Using the procedure of Glick *et al.*(6) to obtain suspensions of pure mast cells from peritoneal fluid of adult albino rats, aliquots containing 960-5200 cells/μl were subjected to the influence of various agents which are either known to influence the morphology and function of these cells, or would be expected to. To 15 μl of a mast-cell suspension in Hanks' balanced salt solution, pH 7.4, 14 μl of agent in solution was added, and after 20 minutes at room temperature, 24°C, 30 μl of 0.01% toluidine blue in Hanks' solution was added. After 10 minutes the cells were examined microscopically in a blood cell counting chamber; percentage of cells degranulated was recorded. The agents used were the following: Heparin sodium (Nutritional Biochemicals), 100 units/mg, in Hanks' soln., pH 7.4. Histamine hydrochloride (Hoffmann-la Roche) in Hanks' soln., pH 7.4. Serotonin creatinine sulfate (Aldrich Chemical) in Hanks' soln., pH 7.4. Hyaluronic acid, human umbilical cord, in Hanks' soln., pH 7.4. Bovine testicular hyaluronidase (Parke Davis) 200 VRU (viscosity reducing units)/mg, in 0.05 M phosphate buffer with 0.05 M NaCl, pH 7.0. Protamine sulfate (Squibb) in Hanks' soln., pH 7.4. Compound 48/80 (Burroughs Wellcome) a condensation product of p-methoxyphenethylmethylamine with formaldehyde, kindly supplied by Dr. Edwin J. de Beer, Tuckahoe, N. Y. *Escherichia coli* endotoxin, Boivin type lipopolysaccharide, prepared and kindly supplied by Dr. Wesley Spink, Dept. Medicine, Univ. Minnesota.

Results. The results are summarized in Table I. The finding that protamine and 48/80 prevent metachromatic staining of mast cells confirms the results of Padawer and Gordon(7) on air-dried and methanol-fixed smears of peritoneal fluid. In our experiments these agents not only prevented staining by toluidine blue, but removed the dye from previously stained mast cells. In both instances the granules were retained. The decolorizing effect is probably due to binding of acid mucopolysaccharide by these agents, as indicated by Mota *et al.*(8). The retention of intact granules is in accord with the finding of Ottoson and Glick(9) that 48/80 produced no demonstrable change in total dry mass per cell as measured by x-ray absorption.

Substances causing edema in mammalian tissues such as histamine, serotonin(10), and *E. coli* endotoxin(11) did not influence the mast cells *in vitro*. This is in agreement with the conclusion of Asboe-Hansen and Wegelius(4,5) that mast cell degranulation *in vivo* may result from tissue edema.

Free extracellular granules, observed in all suspensions of the mast cell, may be due to some mechanical disruption during isolation. In these experiments, as well as in the *in vivo* studies cited, a certain degree of degranulation was observed in buffer or Hanks' solution without agent. A minimum of degranulated cells was observed in the hyaluronic acid solutions.

Testicular hyaluronidase left a spongy or honeycomb-like non-metachromatic structure in isolated mast cells. The heated enzyme lacked this property. When rats were pretreated with an intravenous injection of 2 mg *E. coli* endotoxin 20 minutes before sacrifice, their mast cells had almost no metachromatic granules after hyaluronidase treatment, in contrast to controls. *In vitro,* however, endotoxin did not influence the cells. It should be borne in mind that heparin, not only is unaffected by hyaluronidase, but is an inhibitor of the enzyme. The results lend some evidence to a standing discussion on different

TABLE I. Influence of Various Agents on Mast Cells Isolated from Rat Peritoneal Fluid.

Agent	Final conc. (%)	Influences
Heparin (100 units/mg)	.5	Disintegration of all cells
	.05	*Idem*
	.005	Disintegration of some cells
	.0005	Same as in control Hanks' solution
Histamine	.5	*Idem*
	.05	"
Serotonin	.5	Cell clumping
	.05	*Idem*
Hyaluronic acid	.5	8% of cells partially degranulated
	.05	10% *Idem*
Testicular hyaluronidase (200 VRU/mg)	1.0	76% of cells totally degranulated, no cell disintegration
	.25	60-73% *Idem*
		E. coli endotoxin treated rats: 89-90% of cells totally degranulated, no cell disintegration
Bovine testicular hyaluronidase (65°C, 48 hr)	1.0	36% of cells partially degranulated, same as in phosphate-saline control
Protamine	.5	Cell clumping, no staining, degranulation or disintegration
	.05	*Idem*
Compound 48/80	.5	"
	.05	"
E. coli endotoxin	.5	Same as in control Hanks' solution
	.05	*Idem*
Phosphate buffer, .05 M with .05 M NaCl, pH 7.0		35% of cells partially degranulated
Hanks' solns., pH 3 to 9		31-50% *Idem*

morphological findings on fixed tissue mast cells(12-15), *e.g.*, Morris and Krikos(14) reported no effect by bovine testicular hyaluronidase preparations at pH 5-7 on metachromasia of mast cells in sections of hamster cheek pouch. At low pH the enzyme solutions, whether or not heat inactivated, prevented the metachromatic staining. In mast cells resistant to hyaluronidase, heparin may be exerting its inhibitory effect on the enzyme (1).

Influence of pH was ruled out in our experiments; no morphological changes differing from controls were observed with any of the agents in suspensions of mast cells in Hanks' solution adjusted to pH values from 3 to 9.

Summary. *In vitro* studies on mast cells isolated from rat peritoneal fluid revealed: Metachromatic granules were partially removed by testicular hyaluronidase, but when rats were pretreated systemically with *Escherichia coli* endotoxin, 2 mg iv., this effect of hyaluronidase was much more extensive. *E. coli* endotoxin, 0.5%, did not influence the mast cells *in vitro*. Metachromatic staining of the cells was prevented, and if previously stained the color was abolished, by compound 48/80 or protamine sulfate. These agents and serotonin, 0.5%, neither degranulated nor disrupted the cells, but they produced cell clumping. Heparin, over 0.005% caused cell disintegration, whereas 0.5% hyaluronic acid or histamine did not appear to affect the cells adversely. Hyaluronic acid seemed to protect them to some degree against degranulation.

1. Fulton, G. P., Maynard, F. L., Riley, J. F., West, G. B., *Physiol. Rev.*, 1957, v37, 221.

2. Fawcett, D. W., *Anat. Rec.*, 1955, v121, 29.

3. Riley, J. F., Shepherd, D. M., Stroud, S. W., West, G. B., *Nature*, 1955, v176, 1123.

4. Asboe-Hansen, G., Wegelius, O., *Acta Physiol. Scand.*, 1956, v37, 350; *Nature*, 1956, v178, 262.

5. Wegelius, O., Asboe-Hansen, G., *Exp. Cell. Res.*, 1956, v11, 437.

6. Glick, D., Bonting, S. L., den Boer, D., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 357.

7. Padawer, J., Gordon, A. S., *Anat. Rec.*, 1955, v121, 411.

8. Mota, I., Beraldo, W. T., Junqueira, C. U., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 455.

9. Ottoson, R., Glick, D., *Exp. Cell Res.*, 1958, in press.

10. Benditt, E. P., Wong, R. L., Arase, M., Roeper, E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 303.

11. Thomas, L., *Ann. Rev. Physiol.*, 1954, v16, 467.

12. Compton, A. S., *Am. J. Anat.*, 1952, v91, 301.

13. Hill, M., *Experientia*, 1957, v13, 395.

14. Morris, A. L., Krikos, G. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 527.

15. Velican, C., Velican, D., *Acta haematol.*, 1958, in press.

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Influence of Cooling and CO_2 Content of Blood on Bleeding Time.* (24076)

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Application of a physiological method using hind-limb preparation for studies of bleeding time(1), revealed that certain procedures, such as shorter or larger aeration or more careful and longer refrigeration of blood samples, had an effect on this phenomenon. A systematic study of these effects is here presented.

Methods. As details of the method employing hind-leg of dog for study of bleeding time are in press, including hematocrit, hemoglobin, platelet and clot retraction determinations, we present here a summary of this method. *Hind leg preparation*: Incision of approximately 12 cm on inner side of thigh, beginning at crural arch and following inner edge of sartorial muscle. Dissection of artery, vein and femoral nerve. Isolation of the saphenous branch of the artery and femoral vein, tying off collaterals. Attach 2 threads proximal and distal, to the saphenous branch. Intravenous injection of Liquémine (325 UI Heparin), 0.65 ml/kg of body weight. Plastic tubes were inserted into femoral artery and vein of the heparinized dog and the leg isolated by tourniquet at base of thigh, leaving free the artery, vein and femoral nerve. Arterial blood (citrated) to be tested was forced from a flask by air under variable pressure into the arterial inflow tube and collected as it dripped from the venous outflow. Flow rate was kept constant and controlled by varying flask pressure to maintain an average drop rate of 50 ± 10/minute. Bleeding time was determined on shaved thigh by making 2 shallow razor cuts below incision and wiping away the blood continuously with absorbent paper until bleeding stopped. Blood gas determinations were made with manometric apparatus of Van Slyke(2). For pH determination a direct pH reading meter was used (Electronic Instruments, England). Tests for accuracy were made several times during each experiment with appropriate buffer solution. Blood was aerated by shaking in open flask for half hour. Blood samples previously aerated were restored to normal CO_2 content by agitation 3 hours in flasks containing air plus 9% CO_2. When not in use blood samples were under petroleum-jelly. Blood was refrigerated in environment of water and ice cubes and kept between 1° and 2°C, with occasional stirring, in refrigerator for a half hour; reheating was gradual, leaving blood at room temperature for about half hour, then in water-bath at 37°C, 3 hours. Refrigeration of blood from heparinized dog (1593) and reinjection of this blood after previous rewarming was done as follows: 100 ml of blood was taken from the carotid and refrigerated under petroleum jelly at 1° to 2°C for 15 minutes, then reinjected into saphenous vein after passing a glass coil immersed in water-bath at 40°C. This procedure was repeated every 15 minutes until approximately a whole blood volume of the dog was thus treated.

Results. Cooling Experiments. Normal citrated blood samples from 11 dogs (normal

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TABLE I. Infinitely Prolonged Bleeding Time of Arterial Blood after Aeration.

Aeration time, min.	Blood gases (vol %)				pH		Bleeding time (min.)	
	Under petroleum jelly		After aeration					
	CO_2	O_2	CO_2	O_2	Under petroleum jelly	After aeration	Under petroleum jelly	After aeration
5	23.0	11.6	23.0	13.1	7.55	7.55	9	11
10			16.9	13.3	7.55	7.75	6	+30
30			6.6		7.55	8.15	8	+30
30	19.7	15.9	5.6	18.0			8	+30
30	22.3	15.4	14.7	16.8			7	+30
30	31.9	11.6	18.6	14.8	7.10	7.25	9	+30
30	30.0	10.2	10.6	14.2	7.23	7.66	7	+30
30	30.8	13.5	7.2	16.2	7.25	7.56	9	+30
30							9	+30

values of platelets, hematocrit, hemoglobin and after addition of calcium chloride coagulation and clot retraction time) when cooled for 10 or more minutes at 1° to 2°C lose their previous ability to control experimental skin bleeding in isolated dog's hind-leg preparation. The above phenomenon was confirmed in whole animal when by successive bleedings and cooling of blood samples and reinjection of these samples after previous heating at 37°C, the bleeding time was progressively prolonged. 140 minutes after beginning of the test, the bleeding time was higher than 30 minutes and remained so until end of experiment (4 hours).

Experiments on blood aeration. Table I shows that blood samples from 7 dogs after prolonged aeration, show increase of pH and O_2 content, decrease of CO_2 content and loss of previous ability to control bleeding. If aeration is slight and the blood gas content is not much modified, the loss of hemostatic property is not manifest. If prolonged aeration is followed by adequate exposure to air plus 9% CO_2 (Table II) the pH decreases slightly, blood gas content remains about normal and retains ability to control bleeding. If CO_2 content does not increase to near normal level, after CO_2 exposure, as in one sample shown in Table II, ability to control bleeding is not recovered.

Confirmation that exposure of blood to air and consequent loss of bleeding control is due to a mechanism of liberation of CO_2 is found when blood samples from 12 dogs kept under petroleum jelly or taken into recipient with air plus 9% CO_2 showed a similar behavior, with bleeding times between 5 and 12 minutes when tested in hind-limb preparation.

Discussion. Two alternatives may explain the infinitely prolonged bleeding time after refrigeration. It may be the result of precipitation of an active protein, a substance of the type identified in certain clinical forms as crioglobulins(3), refrigeration being an agent which subtracts from the blood a principle active in haemostasis. An alternative is that low temperature may constitute an exciting factor, causing liberation of substances of antihemostatic character, by elements in blood (probably platelets). The mechanism of this phenomenon is being studied. A different in-

TABLE II. Normal Bleeding Time Recuperation of Aerated Arterial Blood following Suitable Time Exposure to Air plus 9% CO_2.

Time of exposure to air + 9% CO_2 (min.)	Blood gases (vol %)				pH		Bleeding time (min.)	
	Under petroleum jelly		After CO_2 exposure					
	CO_2	O_2	CO_2	O_2	Under petroleum jelly	After CO_2 exposure	Under petroleum jelly	After CO_2 exposure
30	22.5	15.4	14.7	16.8			7	+30
110							7	8
120	26.7	20.0	28.4	17.9	7.20	6.10	6	8
After aeration			7.2	16.2		7.56	9	+30
180	30.8	13.5	32.5	13.5	7.25	7.13	6	8

terpretation must be sought for the prolonged bleeding time when blood is aerated to the point of losing an appreciable amount of its CO_2 content. Since these samples recover their normal bleeding time after shaking in air containing sufficient CO_2, seems to indicate that a particular pH is necessary for the substances concerned in hemostasis.

Observations here reported may guide the research worker, and perhaps guide transfusionist and clinician on the manner of treating blood before employing it as a therapeutic means. In fact, according to experience of workers on platelets, refrigeration of blood as soon as it leaves the donor is essential for good preservation of these elements. However, no particular care is necessary for longer or shorter contact of blood with air. The question of content of CO_2 in blood to be transfused is unimportant, since it will attain its normal gas content in the first passage through tissue capillaries and lungs, although the significance of excessive cooling is very different, since it has been verified that loss of hemostatic property is irretrievable and not recoverable by heating. This could have some practical significance in transfusions to patients with hemorrhagic manifestations.

Summary. Employing the dog's hind-leg which permits a controlled study of bleeding time, it has been determined that refrigeration of blood for 10 minutes at 1-2°C causes it to lose its normal hemostatic properties, not recoverable by heating to 37°C. Furthermore violent aeration of blood in air, by liberating CO_2 and driving the pH to the alkaline side, also renders blood non-hemostatic, although by returning the liberated CO_2 to blood, by shaking in atmospheric air containing 9% CO_2, hemostatic action may be recovered. The mechanism of these phenomena, their application as routine methods in the laboratory, and their implication in technics of blood transfusion are discussed.

1. Cruz, W. O., Oliveira, A. C., *J. Appl. Physiol.*

2. Van Slyke, D. D., Neill, J. M., *Biol. Chem.*, 1924, v61, 523.

3. Lerner, A. B., Watson, C. J., *Am. J. Med. Sci.*, 1947, v214, 410.

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Influence of Environmental Temperature and Chlorpromazine on Biochemical Changes Following Bowel Shock. (24077)

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Several groups of investigators have demonstrated the beneficial action of chlorpromazine* (2-chloro-10-(3-dimethylaminopropyl)-phenothiazine) on survival following hemorrhagic(1,2), traumatic(3), and bowel obstruction(4) shock. Its ability to abolish temperature regulation in animals has been described (5). Evidence for a specific effect upon thermoregulatory centers of the central nervous system is inconclusive; however the apparently complete poikilothermia suggests a primary interference with thermoregulatory centers. The influence of environmental temperature upon survival of normothermic rats following traumatic shock has been evaluated by Erickson, Smith and D'Amour(6).

This report is concerned with the possibility that the beneficial action of chlorpromazine in shock is a result of its effect on thermoregulation, leading in ordinary environmental temperatures to hypothermia and diminished metabolism.

Procedure. White female Holtzman strain rats, weighing approximately 275 g were used. The method for inducing a standard bowel shock was that described by Wendel *et al.*(4). For maximum measurable effects length of obstruction time was 4 hours. Treated animals

* Thorazine hydrochloride (Smith, Kline and French Labs.).

TABLE I. Mean Changes in Rectal Temperatures before and during Bowel Shock Procedure.

	Room temp.	Operation: Before	Operation: After	Release of obstruction	Hours following release of obstruction: 2	4	6	8
Control group	30°	37.0°	36.3°	35.6°	34.4°	33.0°	32.2°	30.6°
	24	36.7	35.5	33.4	30.4	28.2	26.1	25.0
	14	36.8	35.9	33.9	26.2	22.4	20.5	19.0
Chlorpromazine-treated group	30	37.0	35.4	38.2	38.6	38.5	38.7	38.5
	24	37.1	36.5	25.9	25.8	25.3	24.9	24.2
	14	36.8	36.0	16.8	15.8	15.0	14.8	14.6

Each temperature group contained 5 animals.

were administered chlorpromazine intraperitoneally, 12 hours prior to occlusion of the bowel segment, at dose level of 20 mg/kg body weight. Animals from both groups were placed in constant temperature environments of 14, 24, and 30°C immediately following obstruction of the bowel, following release of obstruction, and for 8 hours thereafter. Rectal temperatures were taken prior to and immediately following obstruction of the bowel, at time of release, and every 2 hours thereafter. Heparinized blood samples were obtained 4 and 8 hours following release of obstruction. Blood ammonia was determined by the microdiffusion method of Conway(7); plasma protein by the method of Lowry *et al.* (8); plasma urea nitrogen by the method of Genzkow(9); and plasma glutamic-oxalacetic transaminase by the method of Karmen(10).

Results. Table I presents data showing changes in body temperature of control and chlorpromazine-treated animals kept in the 3 different temperature environments. Table II presents data showing changes in biochemical constituents of blood examined in all groups of animals. Biochemical changes were negligible at lower body temperatures, not entirely because metabolic rate was decreased, but presumably also because of decreased bacterial activity due to considerable cooling of the animal (Table I).

Discussion. Poikilothermic drugs, including chlorpromazine, inhibit in some manner the thermoregulatory mechanism in animals(5) thus requiring the animal to assume the temperature of environment, and to decrease metabolism by 10 to 20%(11). In normothermic animals, survival time following shocking is significantly increased when the animal is kept in environmental temperatures between 17.5 and 25°C(6).

The value of inhibiting metabolic processes of animals in shock has been demonstrated by Kovach *et al.*(12) who administered thiouracil during the shock state and found increased survival time. Conversely, epinephrine stimulation of the metabolic rate reduced survival time. Whether such retardation of metabolic processes is accompanied by a sparing effect on high energy phosphate stores is unknown. On the other hand it has been shown that the adenosine triphosphate content is significantly increased in brain(13), and liver (Wendel and Brophy, to be published) following adminis-

TABLE II. Mean Biochemical Values for Whole Blood and Plasma following Release of Obstruction.

	Room temp.*	NH_3-N, μg/100 ml: 4†	NH_3-N, μg/100 ml: 8	Urea-N, mg/100 ml: 4	Urea-N, mg/100 ml: 8	Protein, g/100 ml: 4	Protein, g/100 ml: 8	PGO-T,‡ units/ml: 8
Control group	30°	422	1349	25.9	5.6	5.69	4.46	193
	24	391	1420	21.6	8.4	5.79	5.20	89
	14	243	277	9.6	10.5	6.56	6.44	50
Chlorpromazine-treated group	30	784	1630	31.8	4.3	5.04	4.81	203
	24	261	454	24.2	31.7	6.11	5.63	46
	14	38	88	12.4	16.8	6.16	6.19	34

* Each temperature group contained 5 animals.
† Hours following release of obstruction.
‡ Plasma glutamic-oxalacetic transaminase.

tration of chlorpromazine. The value of protecting high energy phosphate levels in liver during shock, and its influence on survival have been demonstrated(14).

An increase in plasma urea nitrogen indicates a compensation for increased ammonia production with its subsequent detoxification by the liver, whereas a decrease in urea nitrogen with continued high ammonia levels seemingly indicates liver failure (Krebs-Henseleit cycle). Increased plasma glutamic-oxalacetic transaminase activity indicates liver damage according to Wroblewski and LaDue(15). Decreased plasma protein levels have been observed(16) following induction of shock, thus indicating extensive protein breakdown as a significant factor in aminoacidemia of shock(17).

In the present study, the increases noted in blood ammonia may have been due in part to extrinsic changes in the gastro-intestinal tract, namely increased bacterial activity in the gut. This also was manifested grossly by distention of the bowel segment following obstruction, and by necrosis in bowel wall observed at necropsy.

More interesting, however, is the difference effected by chlorpromazine at 24°C. Here the chlorpromazine-treated animals more quickly assumed the lower environmental temperature, more effectively maintained urea production and blood protein level. At 8 hours after release of bowel obstruction the animals were still actively removing ammonia whereas control animals were not. This lends credence to the suggestion that by this time the controls had already seriously drained the body resources (high energy phosphate?) needed to support this function and that a function of chlorpromazine in the other animals had been to spare these resources by permitting a more rapid development of hypothermia and more rapid reduction in metabolism.

At 30°C the chlorpromazine abolition of body temperature regulation *lessened* the ability of animals to cope with the effects of bowel shock. Here the chlorpromazine-treated animals developed temperatures higher than those of controls, produced more ammonia, and were depleted more rapidly. Thus there is further support for the idea that hypothermia is the important factor for survival in bowel shock. Chlorpromazine is not indicated as a specific survival factor. It has this effect only indirectly, and only if the environmental temperature is below a critical level, to permit development of hypothermia.

Our study demonstrates that bowel obstruction shock is characterized by progressive increases in protein catabolism, probably by most tissues, once the shock syndrome is established, with the release of ammonia into blood. The liver is flooded with ammonia and presumably with amino acids, which are available for deamination and urea formation. At first, the liver seems capable of meeting the demands and urea production is increased, but later it begins to fail and ammonia leaves the liver.

It is thought that 2 mechanisms participated in the bowel shock syndrome, either alone or in union: (1) Following pre-treatment with chlorpromazine, and a lowering of body temperature to that of a hypothermic level, bacterial activity may have been inhibited, thus preventing formation of lethal toxins, toxic amines, ammonia, and other protein breakdown products generally believed to be implicated in the lethal consequences of the shock syndrome. (2) Diminution of metabolic processes consequent to lowered body temperature may have resulted in reduction in amounts of energy expended, thus enabling the liver to detoxify the products of bacterial activity—a sparing effect.

Summary. (1) The extent of biochemical alteration in experimental bowel obstruction shock was related directly and consistently to actual rectal temperatures developed, in both normothermic control rats and those made poikilothermic by chlorpromazine. (2) The value of hypothermia in protecting rats from bowel shock has been demonstrated. (3) Chlorpromazine was beneficial in shock only when its use led to enhancement of hypothermia. Its use at a higher environmental temperature facilitated development of *hyperthermia,* and aggravated the biochemical syndrome of bowel shock. Chlorpromazine ap-

pears to have no specific protective action against shock. (4) Possible mechanisms are discussed.

1. Carruthers, C. F., Gowdey, C. W., *Canad. J. Biochem. and Physiol.,* 1956, v34, 217.

2. Spurr, G. B., Farrand, E. A., Horvath, S. M., *Am. J. Physiol.,* 1956, v185, 499.

3. Cier, J. F., Tanche, M., *Compt. rend. soc. biol.,* 1954, v148, 1834.

4. Wendel, O. W., Griffin, P. E., Brophy, E. M., *Brit. J. Exp. Path.,* in press.

5. Lushbaugh, C. C., Hayes, F. N., Langham, W. H., Ott, D. G., Sanders, P., *J. Pharm. Exp. Therap.,* 1956, v116, 366.

6. Erickson, B. R., Smith, D. L., D'Amour, F. E., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 1.

7. Conway, E. J., *Microdiffusion Analysis and Volumetric Error,* D. van Nostrand Co., N. Y., 1947.

8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., *J. Biol. Chem.,* 1951, v193, 265.

9. Genzkow, C. J., *ibid.,* 1942, v143, 531.

10. Karmen, A., Wroblewski, F., LaDue, J. S., *J. Clin. Invest.,* 1955, v34, 126.

11. Peruzzo, L., Forni, R. B., *Excerpta Med. Sec. II,* 1954, v7, 994.

12. Kovach, A. G. B., Takacs, L., Monacsi, A., Kaldor, V., Kalmor, Z., *Acta physiol. acad. sci. Hung.,* 1957, v11, 181.

13. Grenell, R. G., Mendelson, J., McElroy, W. D., *Arch. Neurol. Psychiat.,* 1955, v73, 347.

14. Rosenblum, D. K., Frank, E. D., Rutenburg, A. M., Frank, H. A., *Am. J. Physiol.,* 1957, v188, 86.

15. Wroblewski, F., LaDue, J. S., *Ann. Int. Med.,* 1955, v43, 345.

16. Anderson, R. S., Stemler, F. W., McHugh, R. F., Rogers, E. B., *Fed. Proc.,* 1957, v16, 3.

17. Russell, J. A., Long, C. N. H., *Am. J. Physiol.,* 1946, v147, 175.

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Control Mechanisms for Trauma Induced DLSH Reaction (Decrease in Mouse Liver Non-Protein Sulfhydryl).* (24078)

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The DLSH reaction is a term used to refer to a statistically significant decrease in liver non-protein sulfhydryl (NPSH) or glutathione (GSH) which occurs within a few hours after subjection of animals to certain experimental procedures. This reaction has been found to occur in mice and/or rats in association with severe trauma(1,2,3), severe cold(1,2,4,5), or administration of toxic or pharmacologic amounts of bacterial polysaccharide(2), insulin(6,7) or epinephrine(8). Register and Bartlett(5,8) have postulated that such a reaction occurring in rats subjected to severe cold, is actually due to stressor activation of the sympatho-adrenal system, particularly to release of epinephrine from the adrenal medulla. Our data indicate that in the DLSH reaction induced in mice by tourniquet trauma, both the sympathetic nervous system and the adrenal cortex play essential roles, whereas the adrenal medulla and the islets of Langerhans do not.

Methods. Mice were made diabetic by injection of alloxan, 100 mg/kg, into the tail vein (cf. Waisbren(9)). In bilateral adrenalectomies each kidney and attached adrenal gland was gently exteriorized through incision immediately lateral to backbone; effectiveness of adrenalectomy was checked in each mouse by autopsy at time of sacrifice. Exteriorization of the kidney and attached adrenal gland also proved extremely useful in adrenal demedullations, performed by an adaptation of the method of Evans(10). Effectiveness of adrenal demedullation procedure was checked for each group of mice by chemical estimation of adrenal medullary hormone content of the pooled adrenals. Tourniquet trauma was performed by the double hind leg ligation method of Rosenthal(11). The ligation period was 2 hours, and sacrifice was usually 2 hours after

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TABLE I. Effect of Insulin on Mouse Liver NPSH and Blood Sugar.

Treatment of mice*					
18 hr fast	Insulin only, subcut.	Insulin & glucose, subcut.	Blood sugar, mg %†	Liver, NPSH, mg %, GSH equiv.†	Insulin action on mice‡
No	No	No	138	249	
Yes	"	"	73	166	
			104	156	
"	Yes	"	20	99	+
			58	105	—
"	No	Yes	16	86	+
			30	103	+
			45	99	—
			63	152	—

* Insulin in amount of 10 units/kg was inj. subcut. 4 hr before sacrifice, *i.e.*, in fasted mice at 14 hr after beginning of fast. 2.5 or 15% glucose was inj. subcut., singly or repeatedly, the total dose being 1.5 mg/kg.

† Values are for samples pooled from 2 or 4 mice.

‡ Convulsions and/or prostration prior to sacrifice.

removal of ligatures. In each experiment, injections and/or hind leg ligations of individual mice were so scheduled that the *average* time of day at which sacrifice occurred was practically the same for all groups. This elaborate scheduling was employed to reduce to a minimum, possible effects of diurnal variation in liver NPSH which occurs in mice and rats (12). P values of Tables were secured by use of Student's t table in Peters and Van Voorhis(13). The methods for estimating liver NPSH and glutathione have been described(12). Blood sugar was estimated by the combined method of Nelson(14) and Somogyi(15). The iodine oxidation method of v. Euler and Hamberg(16) for estimating total medullary hormone content of adrenals was adapted to mouse adrenals, as follows: Adrenals pooled from 10 mice were weighed, and homogenized with 3 ml of 5% trichloracetic acid. Solid material was removed by centrifuging. Fat and trichloracetic acid were removed by 3 separate shakings with ether, about 6 ml/extraction. The adrenal medullary hormones in 1 ml of the extract were completely oxidized, using iodine at pH 6.0 for 3 minutes. A blank not containing adrenochrome was prepared by first mixing all reagents together, so that the iodine was reduced only by thiosulfate, and then adding 1 ml of extract. Finally the optical density of the adrenochrome-containing mixture was read against the blank at 529 mμ using a Beckman spectrophotometer.

Results. Insulin and mouse liver NPSH. Eli Lilly Iletin was diluted with either water, 2.5% or 15% glucose to yield solutions which gave doses of 1, 3, 10 or 30 units/kg when 0.01 ml/g was injected subcutaneously. Four hours later mice were sacrificed and the excised livers were frozen on dry ice until analyzed. For both normally fed and 18 hour starved mice, the minimum dose of insulin required to lower mouse liver NPSH appreciably was 10 units/kg. Extent of insulin induced decrease in liver NPSH was positively correlated with both decrease in blood sugar and likelihood that symptoms of hyperinsulinism would be exhibited (Table I). Glucose *as administered in these experiments* did not prevent hypoglycemia, symptoms of hyperinsulinism, or insulin induced decrease in liver NPSH. Findings made with glucagon-free insulin† were similar to those made with Iletin. Mice injected with insulin-free glucagon† exhibited liver NPSH values within the normal range.

Trauma and liver NPSH of alloxan diabetic mice. Of 18 mice which survived alloxan administration, 7 which exhibited unusually large water intakes were subjected to tourniquet trauma. The average liver NPSH value for these 7 mice was 103 ± S.E. 8 mg% GSH (glutathione) equivalent. Their blood sugar

† Donated by Eli Lilly Co.

TABLE II. Adrenal Glands and Tourniquet Trauma Induced DLSH Reaction.

Operation	Days between operation and exp.	Mean liver NPSH values, mg %, GSH equiv., ± S.E.		P values for significance of differences
		Untreated mice	Traumatized mice	
None		245 ± 10 (6)*	116 ± 7 (8)*	<.0001
		201 ± 9 (7)	116 ± 6 (10)	"
		199 ± 6 (10)	122 ± 6 (10)	"
Sham	0	208 ± 9 (11)	118 ± 4 (20)	"
		204 ± 7 (7)	144 ± 7 (8)	"
	4	272 ± 14 (12)	140 ± 6 (12)	"
Adrenal demedullation	35	193 ± 6 (10)	105 ± 6 (9)	"
		191 ± 5 (9)	112 ± 8 (10)	"
Adrenalectomy	0	204 ± 7 (15)	196 ± 8 (15)	NS
		219 ± 10 (16)	171 ± 9 (14)	.004
	4	215 ± 10 (10)	191 ± 8 (9)	NS
		212 ± 5 (9)	186 ± 12 (10)	"
		230 ± 10 (8)	191 ± 9 (8)	.02
		231 ± 13 (15)	182 ± 14 (12)	.04
	39†	238 ± 9 (10)	231 ± 11 (11)	NS

* No. of livers analyzed shown in parentheses.
† Maintained with fluorohydrocortisone up to 4 days before exp.

values ranged from 300 to 800 mg% glucose equivalent. The remaining 11 mice were sacrificed without being subjected to trauma. The average liver NPSH value for these 11 mice was 237 ± S.E. 14 mg%, GSH equivalent: their blood sugar values ranged from 180 to 590 mg%. It is apparent that a DLSH reaction was induced by tourniquet trauma in mice which had been made severely diabetic by use of alloxan.

Influence of certain surgical procedures on trauma-induced DLSH reaction. Data for 15 experiments are shown in Table II. Trauma-induced DLSH reactions occurring in sham operated and adrenal demedullated mice did not differ appreciably from those occurring in non-operated mice. On the other hand, only slight decreases in liver NPSH occurred in adrenalectomized mice in association with tourniquet trauma. The following data were obtained in analyses for adrenal medullary hormones of pooled mouse adrenals (10 mice/group) by the iodine oxidation method: non-operated mice, 110-130 mg of tissue, about 1 mg/kg of epinephrine (E) plus nor-epinephrine (NE); adrenal demedullated mice 35 days after operation, 60-70 mg of tissue, <0.05 mg/kg of (E) plus (NE) (less than could be estimated by procedure employed).

Effect of l-epinephrine (E) and l-norepinephrine (NE) on liver NPSH of non-operated, adrenal demedullated and adrenalectomized mice. The data (Table III) indicate that the smallest dose of either (NE) or (E), administered over a 5 hour period, which regularly induced a DLSH reaction in non-operated mice was 2.5 mg/kg. The reactions obtained with 2.5 and 5 mg/kg of (NE) and (E) were not as great as those obtained routinely with tourniquet trauma. Higher doses of (NE) and (E) were not tested, since occasional deaths occurred in experiments using (E) at 5 mg/kg. Both (NE) and (E) induced DLSH reactions in demedullated mice. On the other hand, mice adrenalectomized 4 days before experimentation failed to exhibit DLSH reactions on administration of (NE), 2.5 or 5 mg/kg or (E), 2.5 mg/kg. (E), 5 mg/kg proved to be so lethal to adrenalectomized mice that tests at this dosage were discontinued.

Counteraction of trauma-induced decreases in mouse liver NPSH by a ganglion-blocking drug. The drug selected for study was chlorisondamine dimethochloride(17) which exerts a prompt, effective and relatively long-lasting ganglion-blocking action with minimum side effects. Four groups of 10 mice each were set

TABLE III. Adrenal Glands and DLSH Reaction Induced by Adrenal Medullary Hormones.

Operation	Days between operation and exp.	Hormone* and dose (mg/kg)	Mean liver NPSH values, mg %, GSH equiv., ± S.E.		P values for significance of differences
			Saline inj. mice	Hormone inj. mice	
None		NE, 1	198 ± 7 (10)†	176 ±14 (10)†	NS
		5	" "	123 ± 4 (10)	<.0001
		1.25	193 ± 6 (10)	164 ± 8 (10)	.02
		2.5	176 ± 9 (10)	140 ± 4 (12)	.005
		5	186 ± 8 (8)	137 ± 8 (8)	.002
		E, 1.25	171 ± 6 (10)	158 ± 6 (10)	NS
		2.5	" "	140 ± 8 (10)	.02
		5	" "	120 ± 8 (9)	.001
		1.25	193 ± 6 (10)	158 ± 5 (10)	.002
		2.5	176 ± 9 (10)	129 ± 5 (12)	"
Adrenal demedullation	35	NE, 2.5	191 ± 5 (10)	125 ± 7 (9)	<.0001
		5	" "	122 ± 6 (10)	"
		E, 2.5	192 ± 5 (10)	146 ± 6 (10)	.0002
Adrenalectomy	4	NE, 2.5	199 ± 7 (10)	206 ±13 (10)	NS
		5	" "	173 ±11 (9)	"
		2.5	192 ±13 (10)	169 ± 7 (10)	"
		E, 2.5	" "	181 ± 8 (10)	"

* NE = 1-norepinephrine bitartrate; E = synthetic 1-epinephrine neutralized with tartaric acid. Total dose shown was given subcut. in 5 parts at hourly intervals, with sacrifice 1 hr after final injection. Dilutions were made immediately before injections using 0.9% NaCl; all injections were .01 ml/g.

† No. of livers analyzed shown in parentheses.

up. All injections were made intraperitoneally 0.01 ml/g, at 4 and again at 2 hours before sacrifice. Mice of Groups A and B were injected with 0.9% NaCl, mice of Groups C and D with the drug at a concentration of 50 mg% in 0.9% NaCl. The total dose of drug received by mice of Groups C and D was 10 mg/kg. Mice of Groups B and D were also subjected to tourniquet trauma. The liver NPSH values secured, expressed as glutathione equivalents, mg%, ± standard errors, were as follows: Group A (saline controls), 183 ± 10; Group B (saline + trauma), 128 ± 7; Group C (drug controls), 190 ± 8; Group D (drug + trauma), 181 ± 10. It is apparent that the ganglion-blocking drug chlorisondamine dimethochloride practically abolished the trauma-induced decrease in mouse liver NPSH.

Discussion. A trauma-induced DLSH reaction was exhibited by non-operated, sham operated, adrenal demedullated and alloxan diabetic mice, but not to any appreciable extent by adrenalectomized mice, or mice previously injected with the ganglion-blocking drug chlorisondamine dimethochloride. These findings are interpreted to mean that both adrenal cortical and sympathetic nervous system activities are necessary for the DLSH reaction to trauma, but not those of endogenous insulin from the islets of Langerhans or epinephrine (E) plus nor-epinephrine (NE) from the adrenal medullae. DLSH reactions also occur following administration of very large doses of insulin(6,7), E(8) or NE (this paper). Both E and NE mimic trauma, in that following administration of either hormone DLSH reactions occurred in non-operated or adrenal demedullated, but not in adrenalectomized mice. However, E is toxic to the point of acute lethality when used at the minimum dose of 5 mg/kg required to induce a DLSH reaction approaching in size that obtained routinely with trauma; this minimum dose of E is well beyond any dose which the literature indicates could be considered physiological. This fact, coupled with the finding that adrenal demedullated mice exhibit as great a trauma-induced DLSH reaction as do non-operated mice, suggests that a DLSH reaction induced by a very large dose of E or NE may be due to achievement throughout the body of E or NE concentrations approximating those of NE plus E which occur discretely in the

liver in association with intense sympathetic nervous system activity, however induced. This in turn suggests that a DLSH reaction induced by a very large dose of insulin may be due to stressor activation of adrenal cortical and sympathetic nervous system mechanisms necessary to any DLSH reaction, rather than to a specific insulin action on liver NPSH.

Summary. A DLSH reaction (statistically significant decrease in liver NPSH) was secured in mice by administration of very large amounts of insulin, epinephrine or nor-epinephrine, or tourniquet traumatization. Trauma was as effective in inducing a DLSH reaction in alloxan diabetic or adrenal demedullated mice as in normal mice; hence the trauma induced DLSH reaction was not attributable to endogenous insulin or adrenal medullary hormones. The trauma induced DLSH reaction was practically abolished by either adrenalectomy or administration of the ganglion blocking drug chlorisondamine dimethochloride. This indicates that both adrenal cortical and sympathetic nervous system activities are required for the reaction.

1. Beck, L. V., Linkenheimer, W. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 291.

2. Linkenheimer, W. H., Effects of Trauma and Stress on Tissue Sulfhydryl. Ph.D. Thesis, Univ. of Pittsburgh, 1954.

3. Beck, L. V., Linkenheimer, W. H., Marraccini, A., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 823.

4. Bartlett, R. G., Register, U. D., *ibid.*, 1953, v83, 708.

5. ———, *ibid.*, 1954, v86, 397.

6. Binkley, F., Christensen, G. M., Wu, F. C., *J. Biol. Chem.*, 1951, v192, 29.

7. Beck, L. V., Bianconi, A. M., *Fed. Proc.*, 1956, v15, 13.

8. Register, U. D., Bartlett, R. G., *Science*, 1954, v120, 109.

9. Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 154.

10. Evan, G., *Am. J. Physiol.*, 1946, v114, 297.

11. Rosenthal, S. M., *U. S. Pub. Health Rep.*, 1943, v58, 1429.

12. Beck, L. V., Rieck, V. D., Duncan, B., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 229.

13. Peters, C. C., Van Voorhis, W. R., *Statistical Procedures and their Mathematical Bases*, McGraw-Hill Book Co., N. Y., 1940.

14. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

15. Somogyi, M., *ibid.*, 1945, v160, 62.

16. V. Euler, U. S., Hamberg, U., *Acta physiol. Scandinav.*, 1949, v19, 74.

17. Plummer, A. J., Trapolid, J. H., Schneider, J. A., Maxwell, R. A., Earl, A. E., *J. Pharmacol. Exp. Therap.*, 1955, v115, 172.

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Failure of DL-Batyl Alcohol to Prevent Aplastic Anemia in Calves.* (24079)

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This work was prompted by the report of Evans *et al.*(1) that experimentally induced or field cases of Bracken poisoning in cattle could be successfully treated with subcutaneous or intravenous administration of DL-batyl alcohol and comprehensive antibiotic therapy. Hematological aspects and clinical course of Bracken poisoning in cattle are very similar(2) to aplastic anemia induced when certain specimens of trichloroethylene-extracted soybean oil meal (TCESOM) are fed to cattle or calves(3,4). In both instances the intoxication produces a blood dyscrasia characteristic of hypoplastic or, in more severe cases, of aplastic anemia, namely thrombocytopenia, leukopenia, granulocytopenia and a relative lymphocytosis, a moderate degree of anemia, a hemorrhagic syndrome, ter-

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TABLE I. Administration of DL-Batyl Alcohol to Calves with Incipient Aplastic Anemia.

Calf No.	TCESOM fed, lb*	Therapy with DL-batyl alcohol			Result
		Route†	mg, total‡	Started on	
1597	3/4	I.M.	1500	22nd day	Dead 32nd day
1599	3/4	"	"	22nd	" 28th
1623	1/2	"	2500	17th	" 27th
1624	1/2	"	"	14th	" 28th
1625	1/2	"	"	"	Survived§
1629	1/2	"	"	11th	Dead 31st day
1650	1/2	I.V.	2000	"	" 25th
1651	1/2	"	1500	"	" 24th
1680	1/2	I.P.	5000	"	" 26th
1681	1/2	"	"	"	" 28th
1695 & 1697	1/2	" ‖	none	"	" "

* Per day per 100 lb body wt for 10 days only.

† I.M. = intramuscular; I.V. = intravenous; I.P. = intraperitoneal.

‡ 500 mg/day/100 lb body wt on consecutive days except calves 1650 and 1651 which received this amount on the 11th, 12th, 14th, 16th day and on the 11th, 12th and 14th day respectively.

§ See text.

‖ Inj. with solution of Tween only.

minal pyrexia and death. A similar syndrome was recently produced by McKinney *et al.* (5) who administered to calves S-(dichlorovinyl)-L-cysteine, a compound which they synthesized in their search for the toxic factor in TCESOM.

These studies were also of interest in view of claims that batyl alcohol has a therapeutic effect in radiation injury in mice(6) and in patients suffering from irradiation leukopenia (7).

Procedure. Production of aplastic anemia. Aplastic anemia was induced in female Holstein calves, purchased in open market, weighing initially about 90 lb by feeding for 10 days only, ¾ lb or, in most instances, ½ lb/day/100 lb body weight of our standard specimen of TCESOM (referred to elsewhere (8) as TCESOM-6). This was suspended in milk and fed twice daily in equal amounts. The calves were fed milk and alfalfa hay throughout the experiment and during the pre-trial period of 10 to 14 days which was used to assure that the animals were clinically and hematologically normal. Blood counts were made by standard methods(9) at intervals of 2 to 3 days during 10 days of feeding TCESOM and daily thereafter. Specimens of bone marrow were removed for cytological examination by sternal biopsy at least 4 times from each calf and from the ribs when the calves were slaughtered in moribund condition. *Synthesis of* DL-*batyl alcohol.* Methyl- or ethyl-stearate was reduced(10) to l-octadecanol (M.P. 56.5°) and this was converted(11) to the p-toluene-sulfonate (M. P. 56.0°; sapon. equiv. 435.4). This ester was condensed with the sodium derivative of DL-O-isopropylideneglycerol(12) by the procedure(13) with which Baer *et al.* obtained elaidyl-D-O-isopropylideneglycerol(14). The DL-isopropylidene batyl alcohol was hydrolyzed(13) and the DL-batyl alcohol crystallized twice from acetone. The yield of the product (M.P. 71.0°) was as high as 65% based on the p-toluenesulfonate used. Elementary analysis: Found C = 73.0%, H = 12.87%, calculated for $C_{21}H_{44}O_3$: C = 73.2%, H = 12.87%. For injection, the batyl alcohol was solubilized in sterile 5% solution of Tween-80 or Tween-20 in 1% sodium chloride, as described by Evans *et al.* (1); for intramuscular injection of 2 calves, the batyl alcohol was finely ground in an agate mortar and suspended in distilled water. Control animals fed the toxic meal or only milk and alfalfa were also injected intraperitoneally with the Tween solutions to determine their effect on the blood picture.

Results. Production of aplastic anemia by feeding of TCESOM for short periods. Two calves were fed ¾ lb TCESOM/day/100 lb for 7 days only; one was slaughtered in moribund condition on the 32nd day with typical

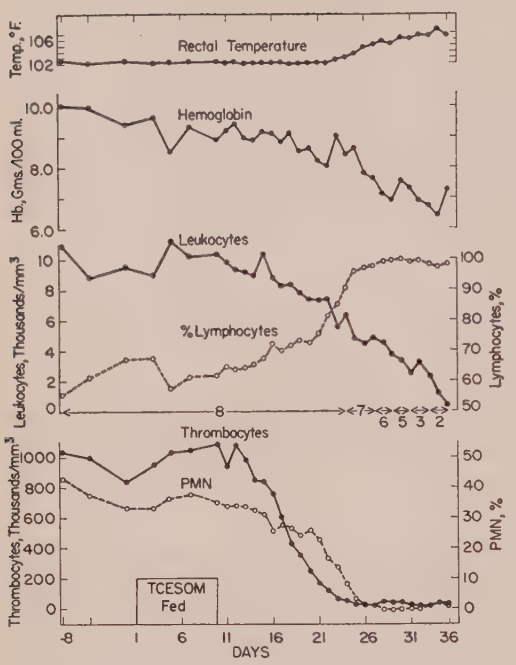

FIG. 1. Changes in cell counts, hemoglobin and rectal temperature during development of aplastic anemia in calves. Aplastic anemia was induced by feeding ½ lb of TCESOM/day/100 lb for 10 days. Each point is the mean of values obtained from the number of calves shown on horizontal line of figure. Decrease in number of calves was due to their death; all calves died.

signs of aplastic anemia, while the other, after temporary severe thrombocytopenia and granulocytopenia, made apparently a complete hematologic recovery and was slaughtered on the 151st day. Feeding of essentially the same total quantity of TCESOM-6 during a 10 day period produced a much higher incidence of fatal aplastic anemia as shown in Fig. 1 and Table I. All calves were clinically normal in every respect until about the 20th day after the start of feeding TCESOM. They consumed their rations eagerly and gained weight. However, as in the case of continuous feeding of the same daily dosage of TCESOM-6(8), after about the 16th day there occurred a rapid decrease of thrombocytes followed by a more gradual decrease of leukocytes. The differential cell counts showed a rapid development of granulocytopenia after the 21st day until virtually all circulating white cells were lymphocytes. Towards the terminal stages there was also a decrease of erythrocytes and of hemoglobin and development of a marked pyrexia. The time sequence of occurrence and the extent of these changes are illustrated in Fig. 1. The mean thrombocyte count of this group of calves, through the 13th day of trial was for unknown reasons higher than usually encountered with calves of this age. This, however, did not delay the onset of severe thrombocytopenia. One calf was slaughtered in moribund condition on the 24th day after feeding of TCESOM had started, whereas 2 survived until the 35th day. There was no distinct difference with respect to onset and course of the blood dyscrasia between calves fed ½ or ¾ lb TCESOM/day/100 lbs.

Microscopic examination of bone marrow biopsy specimens removed prior to about the 18th day after the start of feeding TCESOM failed to reveal morphological abnormalities in the nucleated cells of the erythroid or myeloid series. After that time, however, there occurred a rapidly progressing depletion of all bone marrow cells. In megakaryocytes hyalinization of the cytoplasm was observed as early in the 10th day, followed by progressive disappearance of these cells.

Post mortem examination of calves usually revealed the presence of hemorrhages, primarily in the thoracic cage and the small intestine. In general, however, these were not as extensive, massive and numerous as those found in calves to which the same daily amount of the toxic agent is fed continuously. No external hemorrhages were observed. In this series there also occurred several cases of hemorrhagic consolidation of the apical portion of one lobe of the lungs, with pleural adhesions. This may be related to the respiratory distress experienced by some calves after intravenous injections of batyl alcohol or Tween.

The sequence of events which occurs in development of aplastic anemia when calves are managed as described above, should be particularly favorable for testing the effect of potential therapeutic agents because these can be administered after the toxic compound has been removed from the diet and before the blood dyscrasia has become pronounced.

Administration of batyl alcohol. Table I summarizes the results obtained when 500 mg of DL-batyl alcohol/day/100 lb of body weight was administered by various routes to calves with incipient aplastic anemia. Treatment was started at various intervals after the beginning of feeding TCESOM. In all 10 calves thus treated and in 2 control calves which received only injections of Tween the hematological changes, with respect to time and severity, were essentially those shown in Fig. 1. Eleven of these 12 animals developed fatal aplastic anemia and became moribund between the 24th and 32nd day after the start of feeding TCESOM. One calf (No. 1625, Table I) made a spontaneous recovery from the blood dyscrasia after her blood counts, between the 28th and 31st day had dropped to 20000 thrombocytes/cmm, 1300 leukocytes/cmm and 0% polymorphonuclear leukocytes (for 3 consecutive days) and her rectal temperature had reached 106.5°F. She was kept under observation until the 110th day after the start of feeding TCESOM. By the 50th day the total and differential leukocyte counts had attained normal values; the thrombocyte count reached 700000/cmm between the 59th and 63rd days but subsequently fell again to become stabilized at about 350000/cmm. Since this work has been completed, similar spontaneous recoveries after a near-fatal hematologic crisis between the 26th and 30th day have also been observed with calves injected intravenously, for 10 days only, with 10 mg of S-(dichlorovinyl)-L-cysteine/100 lb/day (unpublished). The amount of TCESOM fed was apparently not quite sufficient to cause 100% mortality. In the other calves treated with batyl alcohol, development and course of the blood dyscrasia were in no way affected by the treatment and no evidence could be found, either from blood counts or from examination of the bone marrow, that hematopoietic stimulation had occurred at any time. Calf 1625 was one out of 12 cases in which the same level of feeding TCESOM was not lethal; her recovery cannot be ascribed to treatment with batyl alcohol.

Two calves which were fed only milk and alfalfa hay were also injected intraperitoneally with Tween-20 for 10 consecutive days; their blood counts remained in the normal range throughout this time and for the following 24 days, after which they were slaughtered.

Intravenous injection of the Tween solutions, with or without batyl alcohol, was not well tolerated by these young calves. Even though injections into a jugular vein were made very slowly, hyperpnea was induced rapidly, followed in several instances by collapse and a period of about 2 minutes when the animals were comatose. Within about 2 hours the animals became essentially normal again. Some calves reacted more severely than others. Because of the severity of the reaction, intravenous injections were not made in the later stages of the intoxication with TCESOM.

Our failure to obtain a curative response with DL-batyl alcohol in TCESOM poisoning must be reconciled with the observations of Evans *et al.*(1) who reported that this compound, together with treatment with antibiotics, had a therapeutic effect in cattle whose blood dyscrasia from Bracken poisoning had reached an acute clinical stage, with associated hemorrhages and pyrexia. Our animals were treated while they were still clinically normal, before such signs appeared. No antibiotics were given to our calves during therapy with batyl alcohol because there was no pyrexia or other evidence of a complicating infection at that time. The total and daily amounts of batyl alcohol administered were presumably at least as large as those (1 g/day to bullocks whose weight was not stated) used by Evans *et al.*(1). It is possible that the toxic agent in TCESOM interferes with different biochemical reactions and at an earlier stage of the development of the affected cell types in the hematopoietic system than the poison in Bracken fern. This cannot be decided until both compounds have been isolated. It is also possible that a relatively large dose of the toxic factor from TCESOM or the induced biochemical defect persist in the animal after consumption has ceased or that an irreversible inhibition of the hematopoietic system is not overcome by therapeutic measures.

Summary. Feeding of ¾ or ½ lb trichlo-

roethylene-extracted soybean oil meal/day/100 lb to young calves for 10 days induced aplastic anemia and caused death 14-25 days after feeding of the toxic material had ceased. DL-batyl alcohol injected by various routes after feeding of the toxic agent had ceased, but before appearance of clinical symptoms, failed to prevent development of aplastic anemia.

1. Evans, W. C., Evans, I. A., Edwards, C. M., Thomas, A. J., *Biochem. J.,* 1957, v65, 6P.

2. Naftalin, J. M., Cushnie, G. H., *J. Comp. Path. Therap.,* 1956, v66, 354.

3. Pritchard, W. R., Mattson, W. E., Sautter, J. H., Schultze, M. O., *Am. J. Vet. Res.,* 1956, v17, 437.

4. Schultze, M. O., Seto, T. A., Mizuno, N. S., Bates, F. W., Perman, V., Sautter, J. H., *Proc.* 6th *Congress, Intern. Soc. Hematol.,* Grune and Stratton, N. Y., 1958, p794.

5. McKinney, L. L., Weakley, F. B., Eldridge, A. C., Campbell, R. E., Cowan, J. C., Picken, J. C., Jr., Biester, H. E., *J. Am. Chem. Soc.,* 1957, v79, 3932.

6. Edlund, T., *Nature,* 1954, v174, 1102.

7. Brohult, A., Holmberg, J., *ibid.,* 1954, v174, 1102.

8. Perman, V., Rehfeld, C. E., Sautter, J. H., Schultze, M. O., *J. Agric. Food Chem.,* 1956, v4, 959.

9. Gradwohl, R. B. H., *Clinical Laboratory Methods and Diagnosis,* C. V. Mosby Co., St. Louis, 1948.

10. Ford, S. G., Marvel, C. S., in Blatt, A. H., Editor, *Organic Syntheses,* 1943, Coll. Vol. II, 372, John Wiley and Sons, N. Y.

11. Marvel, C. S., Sekera, V. C., in Horning, E. C., Editor, *Organic Syntheses,* 1955, Coll. vIII, 366, *ibid.*

12. Renoll, M., Newman, M. S., *ibid.,* Coll. vIII, 502.

13. Baer, E., Rubin, L. J., Fischer, H. O. L., *J. Biol. Chem.,* 1944, v155, 447.

14. Baer, E., Fischer, H. O. L., *ibid.,* 1947, v170, 337.

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Influence of Cobaltous Chloride on Aortic Atheromatosis and Plasma Lipid Pattern in Cholesterol-Fed Chickens. (24080)

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Several investigators have studied the influence of various metallic elements on cholesterol metabolism and experimental atheromatosis in animals(1-8). The action of cobaltous chloride is of particular interest because of its damaging effect on pancreatic α-cells (9). Caren and Carbo(8) found that cobaltous chloride given intravenously causes an increase in plasma cholesterol concentration of rabbits lasting 1 or 2 weeks.

The present paper reports the influence of cobaltous chloride, given in the diet and by subcutaneous injection, on aortic atheromatosis and plasma lipid pattern in cholesterol-fed chickens.

Methods used have been described in detail previously(10). Eight-week-old White Leghorn cockerels were set out in groups of 10 in wire batteries and fed a diet containing 2% cholesterol and 5% cottonseed oil until they were 16 weeks old. Experiments were then terminated. Food was withheld overnight, 4 ml of blood were drawn from alar vein and mixed with citrate solution, the birds were decapitated, and thoracic aortas and brachiocephalic arteries were removed and graded for degree of atheromatosis, using a scale of 0 to 4. Plasma aliquots were analyzed for total cholesterol(11) and lipid phosphorus(12,13), and for cholesterol in lipoprotein fractions separated by Cohn's Method 10(14,15).

Results. $CoCl_2 \cdot 6H_2O$ was given initially in the diet at concentration of 0.5%. It was toxic and accordingly was fed only for the second half of the 8-week experimental period. The cobalt-fed birds in 5 experiments (Table I) had less atheromatosis and lower blood cholesterol concentrations than did the controls. There was no effect on lipid phosphorus concentrations. The treated birds

TABLE I. Lesion Scores, Plasma Lipid Concentrations, and Weight Changes in Cholesterol-Fed Cockerels Given $CoCl_2 \cdot 6H_2O$ in the Diet for 8 Weeks.

Dose, %	Lesions: Incidence	Lesions: Avg score	Cholesterol: Total (mg/100 ml)	Cholesterol: α-Lipoprotein (mg/100 ml)	Cholesterol: β-Lipoprotein (mg/100 ml)	Cholesterol: α/(α+β)	Lipid P, mg/100 ml	C/PL*	Wt gain, g
.5†	24/45	.88	323	54	247	.20	6.35	2.03	342
0	38/47	1.52	525	46	456	.12	6.35	3.18	955
P	.005	<.01	<.001	<.05	<.001	<.001	1	<.001	<.001
0	6/10	1.4	362	34	311	.11	6.22	2.24	1011
.001	7/10	1.25	368	28	322	.12	5.77	2.29	1118
.01	7/10	1.3	281	36	237	.15	5.22	2.08	1116
.05	4/10	.6	293	34	241	.16	4.88	2.46	1000
.25	2/ 4‡	1.0	370	54	289	.20	6.95	2.03	150
.5 †	5/10	.8	257	42	210	.18	5.43	1.81	332
.05	11/30	.42	265	33	217	.16	5.22	2.07	916
0	19/30	1.17	344	35	292	.13	5.66	2.34	990
P	.04	.002	.06	.2	.07	.09	.2	.1	.05

* C/PL = Cholesterol/phospholipid ratio. † $CoCl_2 \cdot 6H_2O$ fed at 0.5% for only 4 wk.
‡ 6 out of 10 died.

generally lost weight during 4 weeks of cobalt feeding, with the result that their over-all weight gain during the 8-week period was only 35% of that of controls. Next, cobaltous chloride was fed in various concentrations in the diet to determine greatest dose that would not reduce weight gain. Four groups of birds were given 0.001%, 0.01%, 0.05%, or 0.25% $CoCl_2 \cdot 6H_2O$ for the entire 8-week period. A fifth group was given 0.5% $CoCl_2 \cdot 6H_2O$ for the second 4 weeks only, and a sixth group, given no cobalt, served as controls. As shown in Table I, small amounts of cobalt caused weight gain, and large amounts caused weight loss and death. Birds fed $CoCl_2 \cdot 6H_2O$ at 0.05% of the diet gained about the same weight as controls, but had less atheromatosis and lower plasma cholesterol concentrations. $CoCl_2 \cdot 6H_2O$ was given at 0.05% in the diet in 2 further experiments. The results of all 3 tests at this level are summarized in Table I. Treatment consistently reduced arterial involvement and lowered plasma cholesterol concentrations. In the second and third experiments the treated groups gained less weight than controls, but the data do not show any tendency within treatments for smaller weight gain to be associated with lower lesion score.

Organ weights. Table II presents the weights of the pituitaries, adrenals, thyroids, and testes, and erythrocyte counts and blood hemoglobin concentrations at the end of one of the experiments with 0.05% $CoCl_2 \cdot 6H_2O$ in the diet. Cobalt-fed birds had smaller pituitaries and testes and somewhat lower erythrocyte counts than controls. The differences in average hemoglobin concentration and thyroid weight were not large enough to be significant. There was no apparent effect on the adrenals.

Examination of pancreas. Pieces taken from 10 birds fed 0.05% $CoCl_2 \cdot 6H_2O$ in the diet for 8 weeks were fixed in Bouin's solution. Sections from head, body, and tail, stained with hematoxylin-eosin and by the method of Gomori(16), showed no evidence of damage to the α-cells. Additional birds were then given cobalt chloride intravenously in single and multiple doses in lethal and sub-lethal

TABLE II. Organ Weights, Erythrocyte Counts, and Blood Hemoglobin in Cholesterol-Fed Cockerels Given 0.05% $CoCl_2 \cdot 6H_2O$ in the Diet for 8 Weeks.

Treatment	Pituitary, mg	Adrenal, mg	Thyroid, mg	Testes, g	RBC × 10^6	Hb, g/100 ml
Cobalt (10)	8.19	174	116	2.73	2.90	9.63
Controls (10)	9.63	177	133	7.71	3.23	10.03
P	.03	.8	.4	.004	.1	.4

TABLE III. Radioiodine Uptake in Cholesterol-Fed Birds Given $CoCl_2 \cdot 6H_2O$, 0.05% in the Diet.

Treatment	Thyroid wt, mg	Thyroid activity, c/m/mg	Blood activity, c/m/ml	Thyroid / Blood
Cobalt (10)	128	802	26.1	35.1
Controls (10)	151	790	24.9	33.4
P	.1	.2	.8	.7

amounts. The single doses ranged from 0.012 mg cobalt chloride to 3.0 mg cobalt chloride. Single doses above this range resulted in immediate death from respiratory failure. Several chicks from each group were sacrificed at 5 hours, 1 day, 3 days, 15 days, and 30 days. One group of 3 chicks was given 4 daily doses of 3 mg cobalt chloride each, intravenously and sacrificed on the 5th day. Again, there was no evidence of damage to the pancreas. As positive controls, 4 guinea pigs were given 3 daily doses of 30 mg/kg cobalt chloride each intravenously and sacrificed 6 or 24 hours after the last dose. Sections of pancreas from these guinea pigs, as well as some normal control animals, were stained simultaneously with those of the chickens. Alpha cell damage was present in the dosed guinea pigs.

Radioiodine uptake test. Damage to the thyroid after oral dosing with cobalt has been observed in man(17). For this reason radioiodine uptake tests were done here on 10 cockerels fed 0.05% $CoCl_2 \cdot 6H_2O$ for 8 weeks and on 10 controls. Each bird was given 1 μc of I^{131} intravenously. Five hours later 4 ml of blood were taken by venipuncture, the birds were decapitated, and the thyroids weighed, placed in Bouin's solution, and counted in a well-type scintillation counter. The results are presented in Table III. The dosed birds again had somewhat smaller thyroids than the controls, and their specific radioactivity was a little higher. The iodine uptakes, as judged by ratio of activity in thyroid to that in blood, were about the same in both groups.

Subcutaneous injection of $CoCl_2 \cdot 6H_2O$ in 3 experiments 5 times a week in doses from 0.005 γ to 25 mg (Table IV) increased both lesion scores and plasma cholesterol concentrations. At highest dose, which killed half the birds, lesion score was reduced, but here again plasma cholesterol was elevated. Inasmuch as birds eat about 80 g of food daily, the 40 mg ingested each day as 0.05% of the diet considerably exceeds the highest tolerated subcutaneous dose.

Summary and conclusions. (1) Feeding of cobaltous chloride to cholesterol-fed chickens reduced the incidence and severity of their aortic lesions and caused blood cholesterol concentrations to be lower than in controls.

TABLE IV. Lesion Scores, Plasma Lipid Concentrations, and Weight Changes in Cholesterol-Fed Cockerels Given Different Amounts of $CoCl_2 \cdot 6H_2O$ by Subcutaneous Injection for 8 Weeks.

Dose	Lesions: Incidence	Lesions: Avg score	Cholesterol: Total (mg/100 ml)	Cholesterol: α-Lipoprotein (mg/100 ml)	Cholesterol: β-Lipoprotein (mg/100 ml)	$\alpha/(\alpha+\beta)$	Lipid P, mg/100 ml	C/PL*	Wt gain, g
γ									
0	6/ 9	1.15	371	39	294	.10	3.58	4.54	813
.005	8/10	1.65	475	42	418	.11	3.70	5.06	948
.05	5/10	1.00	307	34	272	.15	2.83	4.12	996
.5	7/10	1.8	619	47	544	.10	4.30	5.63	855
5	8/10	1.75	404	41	343	.14	3.06	5.25	874
50	9/10	1.3	350	46	297	.15	3.48	4.02	838
0	4/10	.75	291	32	246	.14	4.23	2.57	962
50	6/ 9	1.55	423	37	357	.11	5.63	2.84	853
150	6/ 9	1.17	303	55	233	.20	5.48	2.30	880
1000	8/10	1.45	319	23	273	.10	4.66	2.63	924
mg									
0	5/ 9	.67	222	29	189	.14	4.62	1.96	838
1	6/ 9	1.29	356	31	324	.10	6.39	2.10	912
10	7/10	1.4	386	43	335	.14	7.07	2.13	727
25	0/ 5†	0	451	30	415	.08	6.75	2.67	220

* C/PL = Cholesterol/phospholipid ratio.

† 5 out of 10 died.

The amounts of cholesterol in β-lipoprotein were similarly reduced and $\frac{\alpha}{\alpha+\beta}$ ratios were increased. There was no consistent effect on lipid phosphorus concentrations. Cobalt in the diet did not seem to influence activity of the thyroid. When given either in the diet or intravenously, cobalt did not change the structure of the pancreatic α-cells. (2) Subcutaneous injection of cobaltous chloride generally increased the incidence and severity of aortic lesions and concentration of cholesterol in blood. These findings are similar to those of Caren and Carbo in the rabbit(8). (3) The influence of cobalt in the diet on lipid pattern and aortic lesions was opposite to that of parenterally administered cobalt. It did not produce other effects indicative of previously reported specific systemic actions. Siperstein, Nichols, and Chaikoff(1) concluded that blood-cholesterol-lowering effect of ferric chloride given in the diet was due to precipitation of bile acids, resulting in decreased cholesterol absorption. It is concluded here that the effect of cobalt in the diet of cholesterol-fed chickens similarly is due to a local action in the intestinal tract.

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1. Siperstein, M. D., Nichols, C. W., Jr., Chaikoff, I. L., *Science,* 1953, v111, 386.

2. Curran, G. L., *J. Biol. Chem.,* 1954, v210, 765.

3. Curran, G. L., Costello, R. L., *J. Exp. Med.,* 1956, v103, 49.

4. Mountain, J. T., Stockell, F. R., Jr., Stokinger, H. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 582.

5. Eades, C. H., Jr., Gallo, D. G., *Fed. Proc.,* 1957, v16, 176.

6. Vitale, J. J., White, P. L., Kakamura, M., Hegsted, D. M., Zamcheck, N., Hellerstein, E. E., *J. Exp. Med.,* 1957, v106, 757.

7. Hellerstein, E. E., Vitale, J. J., White, P. L., Hegsted, D. M., Zamcheck, N., Nakamura, M., *ibid.,* 1957, v106, 767.

8. Caren, M., Carbo, L., *J. Clin. Endocrin. and Metab.,* 1956, v16, 507.

9. VanCampenhout, E., Cornelis, G., *Comp. Rend. Soc. Biol.,* 1951, v145, 933.

10. Tennent, D. M., Siegel, H., Kuron, G. W., Ott, W. H., Mushett, C. W., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 679.

11. Abell, L. L., Levy, B. B., Brodie, B. B., Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.

12. King, E. J., *Microanalysis in Medical Biochemistry,* London, J. and A. Churchill, 1951, p67.

13. Zilversmit, D. E., Davis, A. K., *J. Lab. Clin. Med.,* 1950, v35, 155.

14. Cohn, E. J., *et al., J. Am. Chem. Soc.,* 1950, v72, 465.

15. Lever, W. F., *et al., J. Clin. Invest.,* 1951, v30, 99.

16. Gomori, G., *Am. J. Path.,* 1941, v17, 395.

17. Kriss, J. P., Carnes, W. H., Gross, R. T., *J.A.M.A.,* 1955, v157, 117.

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Abnormal Fat Absorption in Tumor-Bearing Rats.* (24081)

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A number of workers have shown that loss of body weight accompanying tumor growth is associated with depletion of total body lipids(1,2). This loss in body weight, however, is not due to anorexia alone, since force-feeding will not overcome depletion of carcass lipid previously described(3). Despite this lipid loss, a progressive, marked hyperlipemia is often associated with tumor bearing in rats when tumor weight exceeds 10% of total body weight(4). Studies of intestinal absorption in cancerous animals have never been described, although absorption may play a very signifi-

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cant role in the above phenomena. Consequently, gastrointestinal absorption of fat in normal, tumor-bearing and pair-fed control rats by use of triglyceride labeled with I^{131} was investigated.

Materials and methods. Three groups of Sprague-Dawley male rats were maintained on isocaloric, synthetic diet complete with salts and vitamins.† The tumor-bearing groups ate *ad libitum* while controls were pair-fed. Water intake was not restricted. Tumors were transplanted by the usual trochar technic into subcutaneous tissue of right inguinal region of rats weighing 130-150 g, using a piece of Walker Carcinoma 256.‡ Tumors were allowed to grow to 15-18% of total body weight, at which time the rats of all 3 groups were placed in restraining cages after thoracic duct cannulation by the technic of Bollman *et al.* (5). All experimental animals had free access to 1% sodium chloride but were not fed throughout post-operative period. Twenty-four hours after surgery, 0.5 ml of I^{131} labeled triolein (Abbott's Laboratories) dissolved in olive oil (total activity 20 μc/ml) was introduced into stomach by gavage, followed by 5 ml of water. Lymph was collected for 6 hours following feeding of radioactive diet. The collected lymph was diluted with 10% potassium iodide and an aliquot of this was mixed with some plasma. The lymph lipids were coprecipitated with plasma proteins upon subsequent addition of 40% trichloracetic acid. The precipitate was washed with 10% KI, and any inorganic I^{131} present was removed (6). Radioactivity of lipid precipitates described above was determined in deep-well scintillation counter (Technical Measurement Co.). The results were expressed in radioactivity in any given sample as percent of total dose administered (%TD).

Results. The percent of total dose found in lymph of normal, pair-fed control and tumor-bearing rats is shown in Table I. These data indicate that tumor-bearing animals absorb considerably less fat *via* the thoracic duct lymph, than do normal and pair-fed control rats. (^{P}tumor, control $<<$ .001; ^{P}tumor, normal $<<$ .001; ^{P}normal, control = 1). Furthermore, it was observed that cannulation eliminated hyperlipemia in both normal and tumor-bearing animals. Therefore, it was concluded that the hyperlipemia of the tumor-bearing rat may be primarily attributed to absorbed, exogenous fat. In view of this, Begg's contention (3) appears valid, that it is unlikely that hyperlipemia of tumor-bearing animals is due to mobilization of endogenous fat. The apparent paradox of excessive hyperlipemia coupled with markedly decreased fat absorption might be resolved by assuming that rate of fat clearance from blood is profoundly reduced in the tumor bearing animal. Reference to this has been made by others (7). The fat absorbed by lymph, then, accumulates in blood and creates the observed hyperlipemia.

TABLE I. Lymphatic Absorption of Dietary Fat Expressed in % of Total Dose (% TD).

Type	Animals No.	Mean body wt, g	% TD in lymph Mean	S.D.
Normals	8	195	15.3	5.1
Controls	5	204	15.4	5.1
Tumor (15-18%)	4	235	5.3	0.5

Loss of carcass lipids may be primarily attributed to marked decrease in fat absorption, and this, coupled with the high energy requirement imposed upon the host by the tumor (8), would account for the cachectic condition of the tumor-bearing animal.

Summary. Absorption of triglyceride labeled with I^{131} in the rat bearing Walker Carcinoma 256 has been studied. The amount of fat recovered from thoracic duct lymph of the tumor rat is significantly less than that recovered from lymph of normal and pair-fed control rats.

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† National Biochemical Corp. Vit. B Complex Diet supplemented with Vit. Diet Fortification Mixture and Salt P-H mixture.

‡ Courtesy of Dr. J. White, Nat. Cancer Inst., Bethesda, Md.

1. Greenstein, J. P., *Biochemistry of Cancer,* Academic Press, N. Y., 1954

2. Haven, Frances L., Bloor, W. R., *Adv. Cancer Res.,* 1956, vIV, 237.

3. Stewart, A. G., Begg, R. W., *Cancer Res.*, 1956, v13, 560.

4. Frederick, G. L., Begg, R. W., *Proc. Am. Assn. Cancer Res.*, 1954, v2, 14.

5. Bollman, J. L., Cain, J. C., Grindlay, J. H., *J. Lab. Clin. Med.*, 1948, v33, 1349.

6. Duffy, B. J., Turner, D. A., *Arch. Int. Med.*, 1958, v48, 1.

7. Begg, R. W., Lotz, F., *Proc. Am. Assn. Canc. Rs.*, 1956, v2, 93.

8. Mider, C. B., *Canadian Cancer Conference* vI, Academic Press, N. Y., 1955.

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Effect of N-Desacetyl Thio Colchicine (TC) and N-Desacetyl-Methyl-Colchicine (MC) on Rat Fetus and Litter *in utero*. (24082)

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TC, when first synthesized by Velluz and Miller(7), had antimitotic activity in non-toxic doses. The single LD-50 for rats, according to Branceni, *et al.*(1) is 175 mg/kg for intraperitoneal doses and 37 mg/kg for intramuscular doses. This is approximately 25 to 100 times less than LD-50 for Colchicine. Rats tolerated daily i.p. doses of 3 mg/kg indefinitely, and 8.5 mg/kg for 21 consecutive days. In preliminary experiments, Jaquier and Feyel-Cabanes (personal communication) found that 10 daily doses of 2.5 mg/kg starting on 2nd, 4th, 8th and 12th day of gestation, had no effect on rat fetuses from 2nd to 12th day, nor when given on 5 consecutive days after 16th day of gestation. TC, however, arrested embryonic development when given beyond 12th day of gestation. Its action appeared directly on the fetus and not on the mothers.

Methods. Experiments were conducted with Long-Evans strain rats bred and reared in our own colony. Only animals which had one satisfactory previous litter were used. Animals were kept on White Breeding diet of Long-Evans, were approximately 6 months old and weighed 250 g each. The compound was given l.p. to groups of rats in different stages of pregnancy. Onset of gestation was determined by massive sperm findings in the vagina. Pregnant animals were kept in single stainless steel cages to avoid urinary or fecal contacts. Mother rats were sacrificed under ether anesthesia on 21st day of gestation. Uteri were removed and contents recorded, weighed and fixed. The fetuses were sectioned or cleared to study skeletal developments. Maternal internal organs were inspected macroscopically, fixed in formalin and studied in H and E paraffin sections.

Results are listed in Tables I and II. TC had no effect on subsequent ovarian cycles or litters when given twice, prior to insemination, to 6 rats (Table II). Doses of 5 mg/kg, given the morning after insemination (day 0) and on day 1 of gestation, were also without effect on litters of 12 rats. Two doses of 7 mg/kg, given on 4th and 5th day of gestation, led to resorption of 23% of fetuses, and complete litter failure in 20% of the experimental group (3 out of 15 rats). Three fetuses were also found dead and 3 others stunted. On 7th and 8th day of gestation, at time of fetal implantation, doses of 5 mg/kg destroyed 34% of all fetuses but only 20% of all litters, whereas 7 mg/kg doses destroyed 65% of all fetuses, and 36% of all litters. On 10th day of gestation a single dose of 1 mg/kg had no effect; 2.5 mg/kg of TC led to resorption of 30% of all fetuses. Only one stunted surviving fetus was found after single 7 mg/kg dose. On 11th day of gestation, a single dose of 5 mg/kg destroyed in one series all litters completely, but in a second series, destroyed only 89% of all litters. However, 2 doses of 5 mg/kg given on 11th and 12th days of gestation destroyed all litters. The 5 mg/kg dose, given on 15th and 16th days of gestation destroyed

* This work was carried out with Grant USPHS and Population Council.

TABLE I. Desacetyl-thio-colchicine, i.p., in Rats.

No. of rats	Day of dose; mg/kg		Mother's wt gain	Uteri total implants	Fetuses					Mothers with comp. litter destruction	Surviving placentas	Litter wt	Fetus	
					Live	Stunted*	Dead	Gross malf.*	Resorb.				Wt	Length
7	0 & 1	a		45	45									
	5 mg/kg	b	21.7	6.4	6.4					0		41.6	6.5	4.5
		c												
12	10	a		100	100									
	1 mg/kg	b	+25	8.3	8.3					0		44	5.3	5.3
		c			100									
10	10	a		79	55				24		22			
	2.5 mg/kg	b	+24.5	7.9	5.5				2.4	0		30.3	5.5	4.2
		c			69.7				30.3		92			
11	11 & 12	a		96	32		1	1	63	4	30			
	2.5 mg/kg	b	+22.7	8.7	2.9	0	.9	.9	5.7		3.4	17.1	5.9	4.5
		c			33.4		1.0	1.0	65.5	36.4	60.3			
10	7 & 8	a		73	48				25	2	10			
	5 mg/kg	b	+28.2	7.3	4.8				2.5		1	25.9	5.4	4.8
		c			65.8				34.2	20	40			
14	11	a		151	0				151	14	112			
	5 mg/kg	b	+14	10.8					10.8		8.0			
		c			0				100	100	74			
36	11	a			23					32				
	5 mg/kg	b	+19.8									30	5.2	4.5
		c								89				
13	11 & 12	a		114					114	13	100			
	5 mg/kg	b	+16.2	8.8					8.8					
		c							100	100	88			
11	15 & 16	a		94	9	1	10	2	73	4	35			
	5 mg/kg	b	+18.5	8.5	.8	.09	.9	.18	6.6		3.1	7.9	4.3	3.2
		c			9.5	11.1	10.6	22.2	77.4	36.4	48			

a = Total b = Per mother c = Percentage of total * Percentage of live.

TABLE II. Desacetyl-thio-colchicine, 7 mg/kg i.p., in Rats.

No. of rats	Day of dose; mg/kg		Mother's wt gain	Uteri total implants	Fetuses					Mothers with comp. litter destruction	Surviving placentas	Litter wt	Fetus	
					Live	Stunted*	Dead	Gross malf.*	Resorb.				Wt	Length
6	2x7 mg/kg	a		50	50									
	mate avg	b	40.3	8.3	8.3					0		45.6	5.5	4.7
	4.4 days	c												
15	4 & 5	a		106	78	3	3		25	3				
		b	+34.5	7.05	5.2	.2	.2		1.6			29.0	4.87	4.55
		c			73.5	3.8	2.8		23.6	20	34			
25	7 & 8	a		208	71	3			137	9	48			
		b	+23.9	8.3	2.8				5.4		1.9	20.0	4.6	3.84
		c			34.2	4.2			65.8	36	35			
3	10	a		28	1	1			27	2	27			
		b	+ 4.8	9.3					9.0		9.0	1.5	1.5	2.9
		c			3.6	100			96.4	66.6	100			
23	11 & 12	a		198					198	23	135			
		b	+13.3	8.6					8.6		5.9			
		c			0	0	0	0	100	100	68.2			
24	15 & 16	a		204			204			24	204			
		b	+22.3	8.5	0	0	8.5				8.5			
		c					100			100	100			
19	18 & 19	a		156			154		2	19	156			
		b	+22.4	8.2	0	0	8.1		.15		8.2			1.3
		c					98.6		1.4	100	100			dead
9	15 & 16	a		85					85	9	72.7			
	prog./50 mg	b	22.8	9.4					9.4		9.0			
		c							100	100	84.7			

a = Total b = Per mother c = Percentage of total * Percentage of live.

87% of all fetuses, but permitted single fetuses, in litter of 7 rats out of 11 animals, to survive. However, doses of 7 mg/kg, given on 11th and 12th, or 15th and 16th, or 18th and 19th days of gestation, destroyed all fetuses of all litters. Two doses of 50 mg of progesterone, given 1 hour before the 7 mg/kg injection of TC, did not protect litters of experimental rats 15 and 16 days pregnant. It is evident that 1 or 2 i.p. doses of 7 mg/kg of TC is lethal to litters from 11th day of gestation onwards. The same doses have less effect on litters at an earlier gestation age, and no effect when given prior to insemination, or at day 0 and 1 of gestation.

Maternal structures. Macro- and microscopically, no effect of TC on the maternal organism was noted in animals treated up to 12th day of gestation and sacrificed on 21st day. Animals treated later showed a mild depression of their bone marrow. This was most noticeable in rats treated on days 18 and 19 of pregnancy. For peripheral blood and bone marrow studies see below.

In many instances, *placentas* survived the resorbed fetuses, especially when rats were treated after midterm. Surviving placentas of fetuses destroyed at time of implantation were often small, measuring only 2-4 mm in diameter, and were partly degenerated. Organs of rats treated at midterm or later corresponded to placentas of control animals of that gestation age. Fetal placental development did not occur in placentas which lost their fetus at midterm. The mammary glands of all experimental rats, who had their entire litters destroyed, even at time of implantation, showed secretory activity comparable to that of normal pregnant controls.

Repeated litter destruction in the same animals with TC. 15 experimental rats, 6 months old and weighing approximately 250 g, who had one satisfactory litter, were mated and on sperm findings in the vagina separated in single cages. On day 7 and 8 of gestation, the rats received, during their first experimental pregnancy, .5 mg/kg TC i.p. and were then remated on day 22, the day of expected delivery and ovulation. Again daily checks for vaginal sperm were made and the animals were injected on days 7 and 8 of the new gestation period, now with 7 mg/kg TC. The dosage was increased because one experimental rat, not contained in this group, produced 2 fetuses after 5 mg/kg doses on days 7 and 8 of her pregnancy. This group of rats were aborted 4 successive times and then remated and permitted to litter. The total average time, in which 4 abortions and post-abortive litters were achieved, was 131 days or 26 days per "pregnancy." The average body weight gain during the period of 4 abortions, *i.e.,* from day 0 of first aborted pregnancy to day 0 of last pregnancy which was not interfered with, was 41%. The breeding performance of rats in their sixth pregnancy (1 pre-experimental litter, 4 abortions and 1 post-abortive litter) was good. The 15 rats had a total of 134 fetuses, average of 8.9 fetuses/mother. Litters had an average weight of 53 g. The average fetus had a weight of 5.9 g, and measured 5 cm in length. Litters were reduced to 3 siblings each and permitted to nurse. After 22 days, the young rats were weaned, put on Long-Evans breeding diet and cross bred. Growth of these rats was normal, as was their breeding performance. No abnormalities were seen by roentgenography on their skeletons or in litters produced.

Experiments with desacetyl-methyl-colchicine (MC). MC, first isolated by Santavy and Reichstein(4) and later synthesized by Uffer, *et al.*(6), inhibited mitosis with less toxicity than colchicine/Cernoch, *et al.*(2). The single LD-100 for the rat was 35 mg/kg, which was 7 times less than the LD-100 for colchicine. Didcock, Jackson and Robson (3) treated pregnant mice and rabbits with varying doses of MC orally and s.c. It interrupted the pregnancy of mice 11 to 13 days pregnant, in doses of 1 to 8 mg/kg s.c. without maternal mortality, but was less effective orally. Rabbits 13 to 16 days pregnant showed interruption of their pregnancy in s.c. doses of 2 to 8 mg/kg, but showed no response to a 2 mg/kg oral dose. The effect of MC was manifested in 1 to 2 days after injection. Our experiments were conducted to study effect of MC on rat litter *in utero* and to compare its action with that of other compounds.

Methods. Groups of pregnant rats, as

TABLE III. Desacetyl-Methyl Colchicine, i.p., in Rats.

No. of rats	Day of dose; mg/kg		Mother's wt gain	Uteri total implants	Fetuses					Mothers with comp. litter destruction	Surviving placentas	Litter wt	Fetus	
					Live	Stunted*	Dead	Gross malf.*	Resorb.				Wt	Length
14	7 & 8	a		123	43	16	2		78	5	50			
	1 mg	b	+24.6	8.8	3.1	1.8			5.7			15	3.1	3.6
		c			34.9	60.0	1.6		63.5	35.7	63.5			
14	7 & 8	a		134					134	14	134			
	2.5 mg	b	+10.6	9.6					9.6		9.6			
		c							100	100	100			
13	7 & 8	a		115					115	13	95			
	5 mg	b	+ 4.2	8.9					8.9		7.3			
		c							100	100	82			
15	11 & 12	a		126	13				113	12	26			
	1 mg	b	+ 8.4	8.4	8.7				7.5			18.5	4.3	3.8
		c			10.3				89.7	80	20.6			
17	11 & 12	a		177					177	17	171			
	2.5 mg	b	+12.7	10.4					10.4		10			
		c							100	100	96.6			
12	11	a		94			94			12	94			
	5 mg	b	+11.2	7.8			7.8				7.8			
		c					100			100	100			
17	15 & 16	a		166			115		51	17	165			
	2.5 mg	b	+ 7.8	9.8										
		c					69.3		30.7	100	99.4			
10	15 & 16	a		93			93			10	93			
	5.0 mg	b	+13.3	9.3			9.3							
		c					100			100	100			
15	18 & 19	a		118	17		97		4	10	101			
	1.0 mg	b	+28.8	7.9	1.1		6.4				6.7	18.0	4.5	4.5
		c			14.4		82.2		3.4	66.6	100	live	0	
25	18 & 19	a		224			224			25	224			
	2.5 mg	b	+11.7	8.95			8.95				8.95	17.1	2.1	2.8
		c					100			100	100	dead		
14	18 & 19	a		113			113			14	113			
	7.5 mg	b	+24	8.1			8.1			8.1	8.1	17.4	2.15	2.9
		c					100			100	100	dead		

a = Total b = Per mother c = Percentage of total * Percentage of live.

described above, received the compound in mg/kg doses on 2 consecutive days i.p., at different periods of gestation. The results are listed in Table III. 1 mg/kg doses led to resorption of 63% of all fetuses, with stunting of 60% of survivors, when given at time of implantation (7th and 8th days). At midterm (11th and 12th days) 90% of all fetuses and 80% of all litters were destroyed with this dosage. The 2.5 mg/kg doses, as well as 5 mg/kg doses, destroyed all fetuses and all litters when given twice after implantation on days 11 and 12, or 15 and 16, or 18 and 19 of gestation. Autopsies of experimental animals treated on gestation days 15 and 16, or 18 and 19 showed, 6 to 12 hours after dose application, fetuses with marked edema, ascites, and hemorrhagic staining of internal organs and outer skin. Microscopically, the organs of mother animals were normal, except bone marrows and intestinal epithelium of rats treated on days 15 and 16, or 18 and 19 and sacrificed within 48 hours. Here 30% depletion of cellular contents of marrow and increased number of mitosis in the crypts of intestinal mucosa was noticeable. No gross abnormalities were seen, but stunting was noted in the few surviving fetuses of rats treated with the 1 mg/kg doses on days 7 and 8 or 11 and 12 of pregnancy. The skeletal ossification centers corresponded to embryos of a younger gestation age and no special bone was singled out in its inhibition.

Bone marrow and peripheral blood studies. Six rats, 200 g in weight, received 7.5 mg/kg TC and another 6 rats received 2.5 mg/kg MC on 2 consecutive days. Two rats of each group were sacrificed under ether and peripheral blood and bone marrow examined at 24, 48 and 72 hours. Hematocrit, hemoglobin, white blood count, differential count, and prothrombin estimation were carried out and bone marrow of sternum and femur was examined. Within 24 hours, the bone marrow of rats treated with MC was fluid, instead of solid and a marked depletion of myeloid elements was noted, with abnormal forms in the myeloid series involving all elements, from polymorphonuclears to myeloblasts. This persisted to 48th hour and was partly repaired at 72 hours. The hematocrit did not fall more than 3 mm, hemoglobin was reduced by 1.2 g and white blood count fell to 5,000, with a depression of polymorphonuclear leukocytes to 1%. TC was found to be less toxic. The marrow depression was less severe and neither hematocrit nor hemoglobin changed. The white blood count showed its greatest depression within 24 hours. The polymorphonuclear leukocytes were much decreased but began to rise after 24 hours. Prothrombin time (Quick's one stage method) of all 12 rats treated varied from 15 to 18 seconds, or from 29% to 48% of normal.

Discussion. TC and MC are used today in chemotherapy of leukemias and gout in man. It is therefore significant that the present data show that the litter of groups of rats have successfully been destroyed with 1 or 2 doses of TC or MC. The price in maternal tissues for the destruction of the litter consisted in a transitory depression of myeloid elements in bone marrow and of polymorphonuclear leukocytes in peripheral blood as well as inhibition of mitosis of epithelial lining of intestinal tract (as already stressed by Branceni, *et al.* (1).) The prothrombin time of our rats was also prolonged indicating a disturbed liver function. Both compounds permitted complete litter destruction without increase in dosage from the time of implantation of fetuses to day before littering. This could not be induced with other antimetabolites without severely intoxicating or sacrificing the mothers (5). Both TC and MC acted on fetuses within hours and induced general edema, ascites and death. In pregnant rats up to midterm, and frequently also at 11th and 12th days of gestation, the fetus would be resorbed with placentas surviving at their developmental stage, corresponding to last living hours of fetuses. In rats pregnant in the second half of their gestation period, the fetuses would stay *in situ* and shrink to a soft gray lifeless and often macerated mass.

The experiment with TC, in which 15 rats were 4 times aborted, was conducted to study the possible permanent effect of TC on fertility, subsequent litters and general intoxication of the mothers. The fact that experimental animals were able to complete 4 gestation periods with 4 abortions and successfully

carry a subsequent normal litter within 131 days is evidence of lack of intoxication. This is supported by an average weight gain of 41.4% of mother animals from onset of first aborted pregnancy to first day of the last carried through litter.

Summary. (1) One or 2 doses of 7 mg/kg of TC or 2.5 mg/kg of MC destroyed the litters of rats when given after 10th day of gestation period. (2) Placentas survived the fetuses. (3) Maternal response to dosage consisted in depression of myeloid tissues of bone marrow, peripheral WBC and prothrombin time. (4) Four consecutive litter destructions *in utero* with TC were well tolerated by a group of rats, did not impair their subsequent fertility or reproduction, nor cause abnormalities in subsequent offspring. (5) The same doses of TC or MC given prior to fertilization, or implantation were less effective in litter destruction.

1. Branceni, D., Peterfalvi, M., Jequier, R., *Arch. Int. Pharmacodyn.,* 1956, v107, 15.

2. Cernoch, M., Malinski, Z., Telupilova, O., Santavy, F., *ibid.,* 1954, v99, 141.

3. Didcock, K., Jackson, D., Robson, J. M., *Brit. Pharm.,* 1956, v11, 437.

4. Santavy, F., Reichstein, T., *Helv. Chim. Acta,* 1950, v33, 1606.

5. Thiersch, J. B., *Acta Endoc.,* Suppl. 28, 37; PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 27, 33, 36, 40.

6. Uffer, A., Schindler, O., Santavy, F., Reichstein, T., Part 3, *Helfa. Chim. Acta,* 1954, v37, 18.

7. Velluz, L., Miller, G., *Bull. Soc. Chim.,* 1954, v21, 755.

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Cytochemistry of Male Reproductive Tract in Scurvy and Inanition.* (24083)

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Investigations have related ascorbic acid to the male reproductive tract. Lindsey and Medes(1) found degeneration of the germinal epithelium in acute scurvy. The inanition which accompanied scorbutus produced the same alterations(1). Goettsch(2) noted similar changes in the testes of scorbutic guinea pigs. Mukherjee and Banerjee(3) observed degeneration and fibrosis of germinal epithelium and Leydig cells in chronic scurvy. They related these alterations specifically to ascorbic acid deficiency rather than to the accompanying inanition. Vit. C studies on animals other than the guinea pig indicate that ascorbic acid is necessary to maintain male reproductive function(4). In the present investigation, cytochemical studies were carried out on the effects of avitaminosis C on the reproductive tract of the male guinea pig, and an attempt was made to differentiate these changes from those which result from inanition in the presence of an adequate dietary intake of ascorbic acid.

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Materials and methods. A total of 80 male guinea pigs were used in this investigation. Each experimental group consisted of 4 animals which were fed as follows: (a) Vit. C deleted diet, *ad lib.*, (b) Vit. C deleted diet, *ad lib.*, plus 10 mg ascorbic acid daily, (c) Vit. C deleted diet plus 10 mg ascorbic acid daily with food consumption adjusted in order that the animal's weight losses would follow those of the scorbutic guinea pig, and (d) commercially prepared Vit. C fortified diet *ad lib.* The animal on the restricted diet (c) was used to differentiate between changes brought about by caloric restriction and those induced by ascorbic acid deficiency. The Vit. C deleted diet was prepared according to Woodruff *et al.*(5) with the following additions: 10 g $CaCo_3$, 2 g K acetate, 40 g Ca pantothenate, 12 mg folic acid and 200 mg ferric citrate per kg of diet. Groups remained on their respective diets until the scorbutic animal appeared

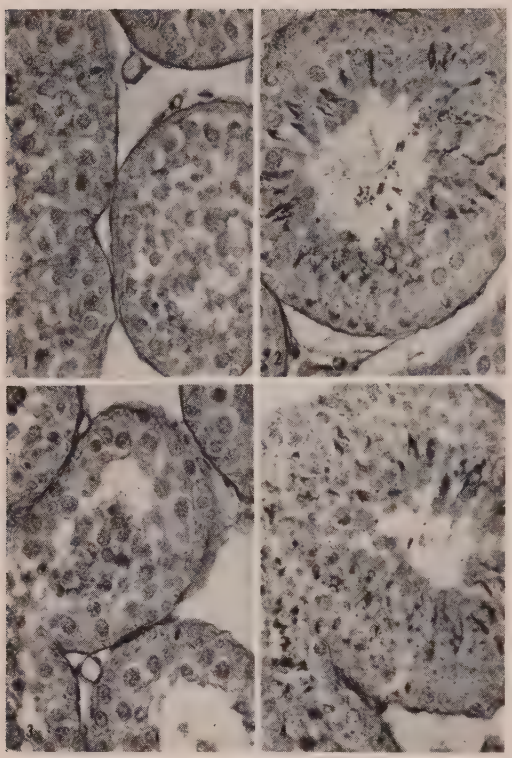

FIG. 1. Scorbutic seminiferous tubules indicating aspermiogenesis. Allochrome staining. ×380.

FIG. 2. Allochrome stained section of dietary control testis. Note presence of developing spermatids. ×380.

FIG. 3. Seminiferous tubules of inanition control animal. Observe absence of spermatids. Allochrome stain. ×380.

FIG. 4. Seminiferous tubule of normal guinea pig. Stained as above. ×380.

moribund showing a typical scorbutic facies, neuritis of the hind limbs, bloody diarrhea, anorexia, malaise and severe weight loss. The time for the development of this syndrome was 25-36 days. Testes were fixed in Orths' fluid and seminal vesicles and epididymides in Lillie's acetic acid alcohol formalin. These tissues were stained by the periodic acid-Schiff technic (PAS) to demonstrate the acrosomic derivatives and differentiate the various stages of spermiogenesis(6). Two types of controls were employed(7). These included: (a) treatment in salivary amylase for one hour at 37°C in order to remove glycogen and (b) staining without previous passage through periodic acid. Reactions were considered positive only if they stained after treatment with periodic acid. The allochrome technic was used to study nuclear morphology and connective tissue elements. In order to demonstrate ascorbic acid, testes, epididymides and seminal vesicles were placed in an acetic acid-silver nitrate alcoholic solution for 30 minutes followed by acid fixation in sodium bisulfite and sodium thiosulfate for 2 hours(8).

Results. Testicular and epididymal morphology. Following application of the PAS reaction to the testes of normal guinea pigs, Schiff-positive staining was localized in the cytoplasm of the interstitial cells, walls of blood vessels, lamellated connective tissue and the basement membrane of seminiferous tubules. Glycogen was present in some of the blood vessel walls. The cytoplasm of the spermatogonia and spermatocytes gave a slight reaction, and numerous brightly staining granules were observed in the latter cells. Throughout spermiogenesis a weak cytoplasmic staining was evident. It was observed that the idiosome was present as a slightly reactive region in the Golgi zone and its granulation was fine and diffuse. With the technics employed it was possible to follow the stages of spermiogenesis in the guinea pig (Fig. 4). Results obtained are in agreement with earlier studies(6). Twelve of the 20 scorbutic animals showed spermiogenic arrest indicated by deficiency of spermatids and spermatozoa in the testes (Fig. 1). Similar changes were noted in 6 inanition guinea pigs (Fig. 3), and 4 of the 8 inanition animals, in which spermatozoa were seen, had large areas of germinal cell aplasia. A single dietary control animal had no spermatozoa and 3 had limited spermiogenic blockage. With these exceptions, the testicular histology of the dietary controls was normal (Fig. 2). Three normal guinea pigs demonstrated aspermiogenesis; however, these particular animals were immature in as much as other pathologic changes consistent with scorbutic or starved animals were not evident. Other indications of spermiogenic arrest in the scorbutic and inanition animals were noted. Nine of each of these guinea pigs had no spermatozoa in the lumen of the ductus epididymis. This was noted in 4 normal animals. Three of these guinea pigs had demonstrated evidence of im-

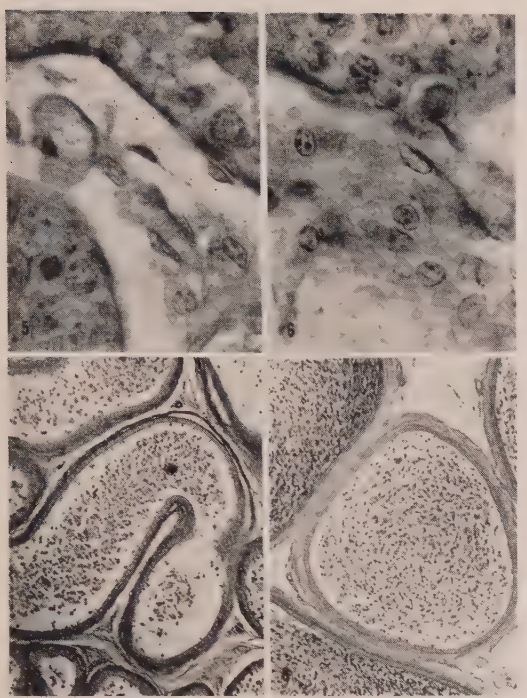

FIG. 5. Leydig cells of scorbutic guinea pig demonstrating pyknotic nuclei and spindling. ×950.

FIG. 6. Leydig cells of normal guinea pig. ×950.

FIG. 7. Cauda epididymidis of scorbutic animal. Note sloughed immature spermatids in lumen of ductus. ×96.

FIG. 8. Cauda epididymidis of normal control guinea pig. Spermatozoa in lumen of ductus. ×96.

maturity on histological examination of the testes. Three dietary controls had no spermatozoa in the ductus epididymis. Sloughed immature and degenerating spermatids were seen in the epididymides of 15 scorbutic guinea pigs (Fig. 7) and 8 inanition controls. This occurred in 2 normal animals and one dietary control; in all 3 of these cases, spermatid sloughing was moderate. Numerous spermatozoa were present in the ductus epididymis of normal guinea pigs (Fig. 8). Pathologic changes in the Leydig cells were noted in 16 of the 20 scorbutic animals (Fig. 5). These cellular alterations consisted of pyknotic nuclei, diminished size and varying degrees of elongation. In some cases, these cells were similar to fibroblasts. These changes were more pronounced in guinea pigs with complete spermiogenic arrest. Half of the inanition animals and 2 dietary controls demonstrated Leydig cell changes. No Leydig cell alterations were seen in the normal guinea pigs (Fig. 6). There were no pathological changes in Sertoli cells, basement membrane or intertubular connective tissue of the testes.

Glycogen studies. Variable quantities of glycogen were localized in the testes, seminal vesicles, and epididymides. There were testicular glycogen deficiencies in the scorbutic and inanition animals. One-half of these guinea pigs had little or none of the substance; the remainder had moderate amounts. Six dietary and 5 normal controls had testicular glycogen deficiencies. The scorbutic animals had more glycogen accumulation in the epithelial cytoplasm of the caput and cauda epididymidis than other controls. Glycogen was increased in the cytoplasm of the seminal vesicle epithelium of scorbutic and inanition animals. Furthermore, there occurred a corresponding decrease in seminal vesicle muscle glycogen in these 2 groups of guinea pigs. Less glycogen was localized in the epithelium and muscularis of seminal vesicles of normal and dietary control animals. There was no glycogen in the Schiff-positive lamina propria of all seminal vesicles studied.

Ascorbic acid studies. Eight scorbutic guinea pigs had no Vit. C demonstrable in the testes and 9 animals demonstrated small to moderate amounts; 8 of these had granules of ascorbic acid in the Leydig cell cytoplasm. One inanition control had no testicular Vit. C and 7 had small amounts. Moderate to large quantities were found in a similar number of inanition animals, one of which lacked ascorbic acid in the interstitial cells. All dietary controls had moderate to large amounts of Vit. C in Leydig cells. Three normal controls had decreased testicular ascorbic acid; Leydig cell Vit. C was present in all members of this group. One-half of the scorbutic animals had no ascorbic acid in the epithelium of the caput epididymidis. Vit. C was localized in the epididymis in moderate to large quantities in most dietary and inanition controls. These 2 groups had more ascorbic acid than the normal animals. Similarly, smaller amounts of Vit. C were noted in the cauda epididymidis of scorbutic and normal animals.

The distribution of Vit. C was random throughout the seminal vesicles of scorbutic and control animals. No differences were noted in the ascorbic acid content of these glands.

Discussion. Spermiogenesis was blocked or inhibited in the majority of scorbutic guinea pigs (Fig. 1) and in many inanition control animals (Fig. 3). The degree of aspermiogenesis was related to the maximum percentage body weight loss in the scorbutic animals. This relationship was not as well defined in inanition controls. Ascorbic acid may offer some protection from the effects of inanition. It is suggested that avitaminosis C results in an inability to utilize other essential dietary substances resulting in more severe cachexia than that noted in the inanition controls.

The greatest amount of ascorbic acid is utilized by the guinea pig during its early growth(9). Previous studies(1-3) used animals not exceeding 300 g in weight. In the present investigation, marked changes occurred in the reproductive tracts of scorbutic and starved guinea pigs weighing less than 350 g, less severe changes being noted in more mature animals. The androgen level may offer some protection from inanition and/or scurvy. Guinea pigs maintained on a regimen intermittently free of ascorbic acid had increased resistance to scurvy(10). These animals may have reached sexual maturity before the end of the experiment.

Vit. C is required for proper formative cell function and has been related to the cellular synthetic processes in that it is in greatest concentration in the Golgi apparatus(11). This is substantiated in the present study by spermiogenic arrest. The proacrosomic granule arises from the Golgi apparatus and subsequently forms the acrosomic granule which is the precursor of the acrosome(6,12). Alteration in the chemical composition of the Golgi apparatus may result in spermiogenic arrest as noted in this study. During inanition Vit. C may be unavailable to its target foci in the Leydig cells or germinal epithelium.

In this investigation only scorbutic animals demonstrated absence of Leydig cell Vit. C. Ascorbic acid may be related in androgen production by these cells. However, nearly all scorbutic and inanition animals had early degenerative Leydig cell change, and Vit. C was abundant in inanition controls. Spermatid sloughing may be associated with inability of scorbutic guinea pigs to produce hyaluronic acid(13). Thus the integrity of the germinal epithelium is not maintained. Appearance of normal Sertoli cells in acute scurvy agrees with most work on tissue changes in experimental scurvy. However, degeneration of these cells has been reported (14).

Epithelial glycogen was present in seminal vesicles of scorbutic and starved animals which had sustained severe spermiogenic arrest. Most normal and dietary controls had abundant testicular glycogen. Scorbutic and inanition guinea pigs had less. Glycogen depletion might result in starvation, but an explanation for seminal vesicle deposition of those same animals is difficult. This may be related to the fructose metabolism of these glands which in turn is an indicator of androgenic activity(15). Thus there is evidence suggesting that scurvy and/or inanition plays a role in reproductive physiology as reflected by these carbohydrate metabolism disturbances in testes and seminal vesicles.

Summary. Histochemical studies were conducted on effects of avitaminosis C on the reproductive tract of the male guinea pig, and an attempt was made to differentiate these changes from those which result from inanition in the presence of an adequate dietary intake of ascorbic acid. Results indicate that scurvy may cause an inability to utilize other essential dietary substances resulting in more severe cachexia than that noted in inanition controls. These studies suggest that changes in the cytochemistry of the Golgi apparatus are involved in spermatogenic arrest. Furthermore, as noted in this investigation, the relation of scurvy and/or inanition to reproductive physiology is indicated by altered carbohydrate metabolism in testes and seminal vesicles. These changes were more marked in scorbutic animals than in inanition controls.

1. Lindsay, B., Medes, G., *Am. J. Anat.*, 1926, v37, 213.
2. Goettsch, M., *Am. J. Physiol.*, 1930, v95, 64.
3. Mukherjee, A. K., Banerjee, S., *Anat. Rec.*, 1954, v120, 907.
4. Phillips, P. H., Landy, H. A., Heizer, E. E., Rupel, I. W., *J. Dairy Sc.*, 1940, v23, 873.
5. Woodruff, C. W., Cherrington, M. E., Stockell, A. K., Darby, W. J., *J. Biochem.*, 1948, v178, 206.
6. Leblond, C. P., Clermont, Y., *Am. J. Anat.*, 1952, v90, 167.
7. Leblond, C. P., *ibid.*, 1950, v86, 1.
8. Lillie, R. D., *Histopathologic Technique and Practical Histochemistry*. The Blakiston Co., Philadelphia, 1954.
9. Reid, M. E., *J. Nutrit.*, 1948, v35, 619.
10. Mellea, J., *Biol. Listy*, 1951, v31, 206.
11. Harris, L. J., *Proc. Nutrit. Soc.*, 1953, v12, 128.
12. Cavazos, L. F., Melampy, R. M., *Am. J. Anat.*, 1954, v95, 467.
13. Penney, J. R., Balfour, B. M., *J. Path. Bact.*, 1949, v61, 171.
14. Bessesen, D. H., *Am. J. Physiol.*, 1923, v63, 245.
15. Ortiz, E., Price, D., Williams-Ashman, H. G., Banks, J., *Endocrin.*, 1956, v59, 479.

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Ciliocytophthoria: Relationship to Viral Respiratory Infections of Humans.*† (24084)

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Papanicolaou(1) described abnormal ciliated epithelial cells found in sputum specimens from some patients with acute and chronic pulmonary disease. The phenomenon was designated ciliocytophthoria, abbreviated as "CCP." The possible significance of CCP as a diagnostic aid in infections of the respiratory tract, and its correlation with the presence or later development of lung cancer were suggested.

The present communication reports observations of sputum cytology, with special emphasis on CCP, in patients with various types of respiratory disease. The results show that CCP is commonly found during pulmonary infections of viral etiology.

Methods. Single specimens produced by deep coughing were collected in 70% ethyl alcohol and smeared and stained by Papanicolaou technic(2). From many patients daily samples were obtained during illness and convalescence. The presence or absence of CCP was ascertained using as criteria the degenerative changes of ciliated epithelial cells described and illustrated in the original report (1). Only those specimens produced by a deep cough (as evidenced in the smear by presence of histiocytes containing carbon particles) were included in the analysis. Smears composed predominantly of squamous epithelial cells and devoid of dust cells were discarded as unsatisfactory. The normal cytogram of deep cough sputum specimens has been described and illustrated by Carabelli (3). Our data were obtained from specimens processed within one or 2 days after sample was received in the laboratory. It may be of practical use to mention, however, that CCP can be demonstrated in positive sputum specimens stored in 70% alcohol at 4°C up to 6 months. Although other cell types are not

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TABLE I. Presence of CCP in Sputum.

Illness	Total No. patients	Total No. patients (CCP positive)
Acute respiratory disease		
Viral influenza	30	26
Primary atypical pneumonia	3	3
Unknown etiology, probably viral	12	11
*Idem**	14	14
Bacterial pneumonia	8	1
Chronic respiratory disease		
Sarcoidosis	24	3
Pulmonary tuberculosis	7	1
Bronchitis	3	1
Asthma	3	3
Carcinoma of lung	2	1
Emphysema and fibrosis	1	0
Non-respiratory disease		
Gastrointestinal bleeding	1	0
Pyelonephritis	1	0
Cerebral thrombosis	1	0
Multiple myeloma	1	0
Rheumatic heart disease	1	0
Myocardial infarction	1	0

* Laboratory personnel (non-hospitalized).

well preserved after prolonged storage, nevertheless CCP is readily apparent. In patients suffering from virus influenza (Asian strains of type A), the etiologic diagnosis was established by studies of other investigators. Their data on identification of viral agents(4,5) and clinical observations(5) are published elsewhere.

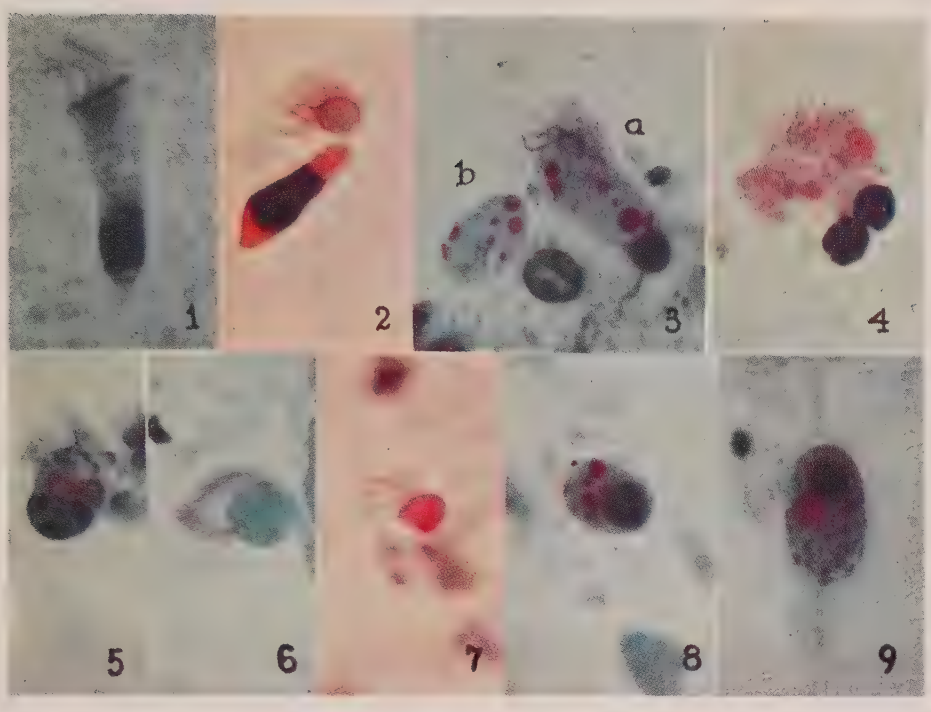
Results. The accompanying photomicrographs illustrate the following abnormal morphological patterns of ciliated epithelial cells observed in CCP positive specimens: nuclear degeneration, pinching off of distal portion of the cell resulting in free ciliated tufts devoid of nuclei, acidophilic inclusions of various sizes and numbers in cytoplasm of the whole intact cell as well as in free ciliated tufts and in remaining basal segments. The acidophilic nature of the inclusion bodies allows them to be easily identified as orange or pink spherical structures within the faint blue or light pink background of cellular cytoplasm.

We emphasize that not all morphological abnormalities described, are found in each positive specimen. The relative proportion of one type to another and its significance in respect to the disease process has yet to be analyzed.

Table I summarizes results of examination for CCP in sputum from patients with various illnesses. It can be seen that CCP was strikingly associated with acute respiratory disease of established or probable viral origin. Specimens from persons infected with influenza virus (Asian strains of type A) showed CCP in 26 of 30 cases. In addition, sputa from the small number of patients presenting the syndrome of primary atypical pneumonia associated with cold hemagglutinins all revealed CCP. Specimens from patients suffering from acute respiratory infection of probable viral origin were positive for CCP almost without exception; in these individuals the clinical pattern of the illness (insidious onset, constitutional symptoms, absence of pneumonic infiltration and of leucocytosis, and recovery without antimicrobial therapy) suggested strongly a viral causation, but all efforts to establish an etiologic diagnosis were without avail. Similarly CCP was present in sputa from non-hospitalized laboratory personnel exhibiting symptoms suggestive of a viral respiratory infection.

In contrast, CCP was found rarely in sputum from patients with bacterial pneumonia, or with various chronic respiratory diseases. Some of the rare CCP positive specimens in these groups were obtained from patients in whom an intercurrent viral infection was suspected. CCP was not observed in sputum from a small number of patients with diseases of other organ systems.

Table II demonstrates the presence of CCP in serial sputum specimens from patients infected with Asian strains of influenza virus (type A), as established either by virus isolation, or by demonstration of rising serum antibodies, and in many instances by both methods. In each of these cases, specimens obtained during the first few days of illness were comprised predominantly of CCP and mononuclear cells. The engulfment of ciliated tufts or basal fragments of CCP by histiocytes was occasionally observed. In those cases which ran an uncomplicated course, CCP persisted during the first week of illness; sputum smears made during subsequent convalescence often showed numerous large, multinucleated histiocytes with orange or pink cytoplasm.



PHOTOMICROGRAPH 1. Magnification × 1100. Morphological Changes Characteristic of Cilio-cytophthoria (CCP).

1. Normal ciliated cell.

2. Pinching off of ciliated portion and nuclear change of basal segment.

3. (a) Pyknotic nucleus and inclusions of various sizes in cytoplasm of intact cell.
(b) An anuclear segment containing inclusions.

4. Two cells undergoing disintegration. Note degenerative nuclei and large inclusions in cytoplasm.

5. Nuclear change with characteristic chromatin grouping in intact cell.

6 and 7. Free ciliated tufts.

8 and 9. Basal segments containing pyknotic nuclei and cytoplasmic inclusions.

The photomicrographs were taken by Mr. Julian Carlile of The Rockefeller Institute.

TABLE II. Duration of CCP in 13 Patients with Viral Influenza.

Presence of CCP in sputum (days after onset of illness)															
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
+	+	+	+	+											
+	+	+	+												
+	+	u.s.	u.s.	+	+										
	+	+													
		+	+	+	+	u.s.									
		+	+	+	+	+	+	+	u.s.				0		
			+	+		u.s.				u.s.					0
			+	+	+										
			+	+	0										
		+		+	+			+							
			+	+	+	+						0			
			+	+	u.s.	0			+			0	+		0
			+	u.s.	0	0			+		0		0		

Blank space on chart indicates specimen not received. (u.s.) indicates unsatisfactory specimen.

Specimens identified in descending order as follows: P.C., L.W., E.T., E.C., M.B., Tuc., V.G., Cou., Mur., Rui., Lah., Ger., Nad.

Of special interest were observations on several influenza patients who developed complicating bacterial pneumonia. The development of bacterial pneumonia coincided with a striking change in cytological pattern of the sputum smear, the cytogram changing from that of CCP and small and large mononuclear cells seen during initial influenza to a picture comprised predominantly of polymorphonuclear leucocytes during the bacterial pneumonia.

Discussion. The present studies were undertaken primarily to gain information about the significance of a particular type of degeneration (ciliocytophthoria) observed in exfoliated ciliated epithelial cells in certain sputum specimens. Is the occurrence of CCP the result of specific damage to the respiratory tract, or do these abnormal cellular changes appear in shed ciliated cells regardless of the factor producing exfoliation? Occasional columnar ciliated cells were often present in specimens from patients with acute bacterial bronchopulmonary disease or various chronic lung conditions, yet the degenerative changes of CCP were not seen in these instances by us or by others(3,6). Thus, it is suggested that there is, indeed, a certain specificity associated with CCP.

On the other hand, CCP was observed in smears of sputum obtained from patients with a variety of acute viral respiratory infections. Further studies are required to determine whether or not the numerous viral agents which infect the tracheobronchial tree differ one from the other in respect to the quantity or type of CCP which they incite.

Previous workers have described abnormal changes in ciliated respiratory epithelium accompanying viral influenza infection and have suggested the usefulness of these observations in establishing a tentative diagnosis(7,8). In addition, Hers(7) has observed similar degenerative changes in ciliated cells in specimens obtained from fatal cases of measles and varicella bronchopneumonia, and has suggested that these alterations are not specific for influenza but represent infection with epitheliotropic viruses in general. The fact that inclusions characteristic of CCP were not described by Hers(7) may be due to the different technics of staining employed. Inclusions in tracheobronchial epithelium obtained at autopsy from cases of varicella pneumonia have been described by others(9,10).

The number of specimens available to us from patients with established pulmonary carcinoma was insufficient to permit evaluation of a possible relationship between CCP and lung neoplasms. For the same reason, the presence of CCP in bronchial asthma cannot be properly assessed. It is possible that the asthmatic attacks in the 3 cases studied may have been provoked by viral infections. CCP has not been described by others who examined bronchial aspirates or sputum specimens from a large series of patients with asthma(3,6).

There is great need at present for an ac-

curate method of making a prompt diagnosis in respiratory infections. Although it is well recognized that a high proportion of acute illnesses accompanied by cough and fever are of viral origin, therefore not requiring the administration of antimicrobial agents, the physician is usually forced to decide on specific treatment before results of bacteriological and virological examinations are available. Therefore, antibiotic therapy in acute respiratory infections is commonly instituted empirically. The results of our investigation suggest that cytological examination of sputum may be helpful in prompt diagnosis of acute pulmonary infections. In addition to the clinical pattern and other laboratory features of illness, the presence or absence of CCP in sputum from a satisfactory deep cough specimen provides a diagnostic aid which may serve to distinguish between infections of viral and bacterial etiology.

Summary. 1) Sputum specimens from patients with acute and chronic pulmonary diseases were examined cytologically by the Papanicolaou technic. Certain abnormalities in exfoliated ciliated epithelial cells (ciliocytophthoria) were observed in specimens from a high proportion of individuals with viral respiratory infections; in contrast, ciliocytophthoria was seen only rarely in sputum preparations from patients with acute or chronic pulmonary disease of non-viral etiology. 2) Sputum cytology may prove valuable for prompt differential diagnosis between viral and bacterial respiratory infections.

1. Papanicolaou, G. N., *New York State J. Med.,* 1956, v56, 2647.

2. ———, *Atlas of Exfoliative Cytology,* Harvard University Press, 1954.

3. Carabelli, A. A., *Am. Rev. Tuberc. Pul. Dis.,* 1958, v77, 22.

4. Choppin, P. W., Osterhout, S., Tamm, I., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 513.

5. Rogers, D. E., Louria, D. B., Kilbourne, E. D., *Trans. Assn. Am. Phys.,* 1958, in press.

6. Brumfitt, W., Willoughby, M. L. N., *The Lancet,* 1958, v1, 132.

7. Hers, J. F. Ph., *The Histopathology of the Respiratory Tract in Human Influenza,* H. E. Stenfert Kroese N. V., Leiden, 1955.

8. Pigarevski, V. E., *Acta Virol.* (English Edition), 1957, v1, 30.

9. Waring, J. J., Neuberger, K. T., Geever, E. F., *Arch. Int. Med.,* 1942, v69, 384.

10. Cheatham, W. J., Weller, T. H., Dolan, T. F., Dower, J. C., *Am. J. Path.,* 1956, v32, 1015.

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Superior Olive in the Alligator.* (24085)

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Rasmussen(3) described for the cat peduncular fibers which leave the central nervous system between the 2 divisions of vestibular nerve and dorsal to rootlets of the pars intermedia. These fibers then course in the inferior division of the vestibular nerve as far as the ganglion associated with the main saccular ramus to the cochlear nerve. His findings were based on experimental material studied by the Marchi method(4) and by chromatolytic methods. Rasmussen(3) states that it was "extremely difficult, if not impossible, to determine the exact cells of origin of the peduncle by the Marchi method" but that it was "possible to delimit a definite area of origin with the aid of small lesions variously placed. The possibility of peduncular fibers passing through the olive from elsewhere was ruled out by circumscribing it with lesions." After section of the eighth nerve he found, in cresyl violet stained sections, no significant alterations or cell loss in the superior olivary complex proper, but on the contralateral side many retro-olivary cells appeared shrunken.

* I desire to express my appreciation to Dr. George Clark for his gracious counsel on the problem, and to Dr. C. Willet Asling for helpful suggestions regarding final draft of paper.

Because of inconsistencies of the method and few cases studied, his results by the chromatolytic method could be considered valuable only to the extent of indicating an agreement with conclusions reached by the Marchi method. This olivocochlear tract of Rasmussen is apparently also present in the alligator (*Alligator mississippiensis*).

Methods. In work reported separately (1, 2) various branches of the facial nerve were cut in 25 alligators; the entire facial nerve was sectioned intracranially in 4, and both facial and auditory nerves were sectioned in one alligator. Forty-three days after operation, the animals were sacrificed. The brains were exposed and placed immediately in 95% alcohol. Several hours later the medullae were removed from cranial cavities and placed in fresh 95% solution of alcohol for 48 hours. The tissue was then dehydrated, embedded, and sectioned. Serial sections, 10 μ thick, were cut and stained with 0.5% toluidine blue in aqueous solution overnight. The sections were then differentiated in 95% alcohol and counter-stained with erythrosin. Serial sections of 2 normal alligator brains were cut; one was stained with toluidine blue and counterstained with erythrosin; the other was stained with Mallory's phosphotungstic haematoxylin.

Results. In the alligator which had both VIIth and VIIIth nerves sectioned, there were chromatolytic changes in most of cells of the superior olive contralateral to sectioned VIIIth nerve.† These altered cells were similar to those evident in the motor nucleus of VIIth nerve homolateral to the section. The former were larger than normal and stained a homogeneous pink. No chromatolytic changes contralateral to site of section were seen in any animal in which the VIIIth nerve was intact.

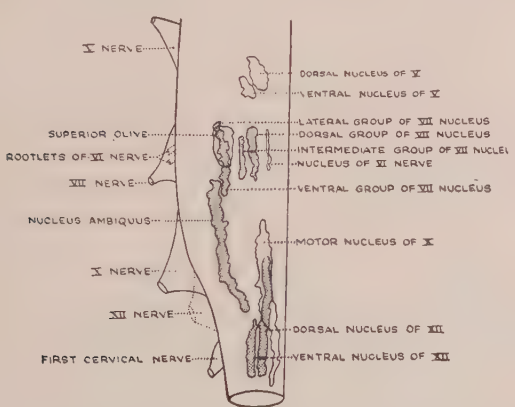

FIG. 1. A graphic reconstruction of lateral half of medulla oblongata showing superior olive and its relation to motor nuclei in alligator. It is based on serial sections reconstructed by projection on the horizontal plane.

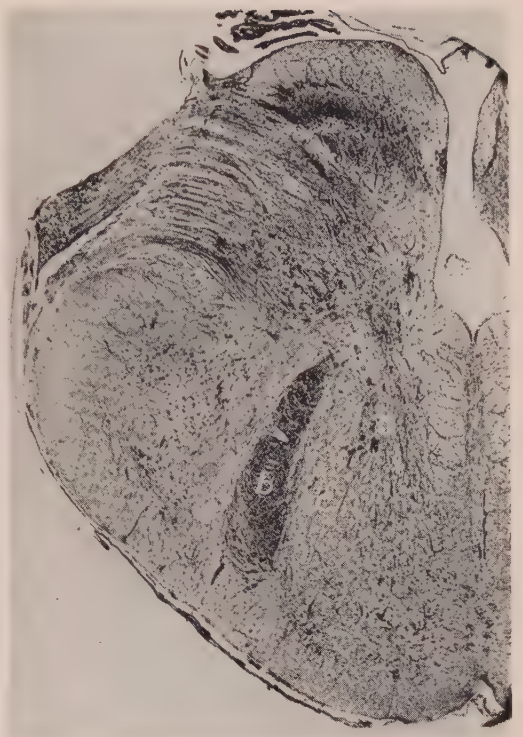

FIG. 2. Cross section of brain of alligator 43 days after motor part of seventh nerve was cut. This section shows superior olive lateral to the dorsal and intermediate parts of the nucleus of seventh nerve (× 20). a. Dorsal part of the nucleus of nerve VII. b. Superior olive. c. Intermediate part of the nucleus of nerve VII.

When the superior olive was projected on a horizontal plane from stained cross sections, it was roughly oblong in shape and closely related to the several parts of the facial nucleus (Fig. 1 and 2). The lateral group of the facial nucleus is adjacent to the ventrolateral

† (a) The undamaged ones might be related to the bundle connecting to the reticular formation and medial longitudinal fasciculus. (b) A detailed study of Nissl changes due to axone reaction in the alligator is now in progress.

part of the superior olive; the ventral group lies adjacent to the ventromedial part of the superior olive; the intermediate cell group lies between the dorsal and ventral cell groups and medial to the superior olive; while the dorsal cell group lies between the nucleus of VIth nerve medially and the superior olive laterally.

In normal material stained with Mallory's phosphotungstic haematoxylin, fibers can be seen coursing dorsally along the medial and lateral sides of the superior olive and then medially toward the mid-line. In all probability these represent fibers from the cells of the superior olive. It was impossible to trace these fibers with certainty to the vestibular nerve by this method. However, chromatolytic changes could be demonstrated in the superior olive of the opposite side following the auditory nerve section; therefore, it seems plausible to assume that fibers of the superior olive course with fibers of the vestibular nerve in the alligator exactly as Rasmussen has described in the cat.

Summary. 1. When the entire facial nerve of the alligator was transected intracranially, there were chromatolytic changes in the facial nucleus, but none in either superior olive. 2. When both the facial and auditory nerves were sectioned, chromatolytic changes were particularly noticeable in the contralateral superior olive. This indicates that the olivocochlear bundle which Rasmussen has described in the cat is also present in the alligator.

1. Gillaspy, Carrie C., *J. Comp. Neur.*, 1954, v100, 481.

2. ———, *Anat. Rec.*, 1955, v121, 397.

3. Rasmussen, G. R., *J. Comp. Neur.*, 1946, v84, 142.

4. Swank, R. L., Davenport, H. A., *Stain Tech.*, 1934, v9, 11.

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A Factor in Serum of Human Beings and Animals that Destroys *T. vaginalis.* (24086)

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When a pure culture of *Trichomonas vaginalis* (Donne 1836) was injected into the anterior chamber of eyes of rabbits, the parasites disappeared rapidly and cultures of the aqueous humor became negative for parasites after 5 to 30 minutes(1). Since aqueous humor itself has been shown not to be trichomonicidal (unpublished), it was thought that the parasites were probably cleared from the anterior chamber through canals of Schlemm and eventually reached the blood stream. To learn more about the fate of the parasites in the blood stream, pure cultures of *T. vaginalis* (8-10 million motile organisms) were injected into the veins of 2 rabbits and cultures of the blood and various organs taken. Cultures for *T. vaginalis* taken within 10 to 15 minutes after inoculation showed that the bloods were sterile and that virtually all organs were sterile. Although the reticulo-endothelial system probably was active in ridding blood of many organisms, the possibility was considered that a large number of parasites were killed by the blood itself. Experiments demonstrated that human and animal serums have a remarkable ability to destroy *T. vaginalis* and that this lethal activity is lost when serum is heated at 56° C for 30 minutes.

Materials and methods. Organisms. Two strains of *T. vaginalis* isolated from 2 patients with vaginal trichomoniasis were employed; they reacted similarly in all studies. At first, 2-day cultures grown on thioglycolate horse serum medium with antibiotics (T.H.A.)(2) and containing about 2 million organisms/ml were used. In later experiments, cultures were

concentrated by centrifugation so that 1 ml contained approximately 4 million parasites. *Serums.* A total of 54 fresh serums from unselected human beings and from various normal animals were tested for trichomonicidal activity. These included 18 human serums, and serums from 10 guinea pigs, 5 rats, one pool from 4 hamsters, 4 serums from 2 dogs, and 16 serums from 9 rabbits. Controls consisted of the same serums heated in water bath for 30 minutes at 56°C. *Importance of pH on trichomonicidal activity of serum.* Early in this work it became apparent that pH of a serum was an important factor in the tests to be described. pH of a serum rises when stored over-night at 4°C; simultaneously the disintegrating effect of serum for the parasite is reduced. To prevent loss of CO_2 with consequent rise in pH approximately 4 ml of blood were distributed into 10 x 1.2 ml tubes and the tubes immediately plugged with rubber corks. After remaining at room temperature 1 to 2 hours, they were placed in refrigerator at 4°C over-night. The clot was rimmed and the air over clotted blood was replaced by 5% CO_2 in nitrogen gas. The tubes were immediately closed with rubber corks and centrifuged at 1500 rpm for 20 minutes. The serums obtained from the clots were kept in ice water bath while being tested. Surplus serum was stored at 4°C after replacing air over serum with CO_2. In this fashion most of the disintegrating activity of serum for *T. vaginalis* was maintained after storage for 2 weeks at 4°C. The pH of mixtures of equal parts of culture and serum was approximately 7.2.

Results. Lethal effect of fresh serum on T. vaginalis. Fresh rabbit serum and the same serum heated to 56°C for 30 minutes were diluted 1:2 and 1:4 with thioglycolate medium (without horse serum and antibiotics added). To 1 ml of each tube, 0.05 ml of thioglycolate medium and 0.05 ml of the culture were added. After incubating all tubes in water bath at 37°C for 30 minutes or 1 hour, 0.5 ml or 1 ml of the contents of each tube was cultured in T.H.A. Cultures of 1:2 and 1:4 dilutions of fresh serum and the parasites were negative for *T. vaginalis,* whereas cultures of heated serums and parasites, and culture control were in every case positive within 24 hours. This experiment was repeated with serums from 2 other rabbits and one dog with similar results and demonstrated that fresh serum killed *T. vaginalis* while controls with heated serum had no such effect.

Morphologic changes in parasite when exposed to serum. Three variations of direct, warm stage, microscopic examination of mixtures of the parasite and serum were used. *First,* 2 drops of chilled serum and 2 drops of culture were mixed directly on a slide; *second,* equal volumes of culture and chilled serum were mixed in a tube and 4 drops of this mixture immediately transferred to a slide; *third,* equal volumes of cultures and chilled serum were mixed in a tube, incubated at 37°C in water bath and 4 drops transferred to a slide after intervals of 1 to 5 minutes or longer. Controls with "inactivated" serum were made. The 3 variations of technic gave essentially the same results with 50 different human and animal serums tested.

Microscopic examination of the mixtures of serum and cultures revealed that the parasites first lost their motility, then suddenly appeared indistinct and flattened out, forming floating shadows or ghosts. The shadows gradually disintegrated and in 5 minutes or less virtually disappeared, leaving only a few indistinct forms and granular debris on the slide. The progressive transformation from the motile organisms to complete dissolution appeared identical for all serums tested, but the time required for destruction of the parasites varied according to the species from which the serum was obtained. Thus, the parasite was usually completely lysed within 3 minutes in dog and rat serum whereas it usually required 4 or 5 minutes for the parasite to be lysed by human, rabbit, guinea pig, and pooled hamsters' serums. In general the potency of a serum appeared to be inversely proportional to the time required for disintegration of the parasite. These destructive effects were not observed in controls. In the preparations of serum that had been heated to 56°C for 30 minutes plus parasites, the organisms remained motile for 15 to 20 minutes, at which time the preparation began to dry and the parasites became distorted without forming shadows or ghosts. Several of the

heated serums agglutinated the organisms slightly but such agglutination did not interfere with motility of the parasite. The preparation of parasites diluted with thioglycolate medium gave results similar to preparations of heated serums and parasites except that agglutination did not occur.

Discussion. Nuttall(3) and Buchner(4) were the first to demonstrate that normal blood and serum were bactericidal for certain bacteria. Nuttall(3) observed that the bactericidal property of normal serum was destroyed by heating at 56°C for 30 minutes. For many years thereafter "complement," a term used by Ehrlich(5) for the heat labile substance together with natural antibody, was considered the only bactericidal substance in normal serum. Recently, however, Pillemer and his associates(6) have demonstrated in normal human serum and other serums a substance which they named properdin. In the presence of complement and Mg++, properdin participates in bactericidal, virus-neutralization and hemolytic activities. Properdin, like the factor in serum which destroys *T. vaginalis,* is also thermolabile. Feldman (7) has reported a toxoplasmal antibody activator in the serum-properdin system. Recently Borsos and Warren (Am. Soc. Tropical Med. 1957) described 2 factors in serum of adult chickens; the first was thermolabile and lysed *Trypanosoma cruzi,* the second was thermostabile and agglutinated this parasite.

This paper is limited to the demonstration that normal serum from human beings and various animals kills and disintegrates *T. vaginalis,* and that this ability is lost after heating at 56°C for 30 minutes. In current studies attempts are being made to determine the nature of the active substance and its effect on other parasites.

Summary. Human serums and serums from several species of animals have the capacity to kill and disintegrate *T. vaginalis* with great rapidity. This activity of serum is destroyed by exposure of the serum to 56°C for 30 minutes.

1. Weld, J. T., Kean, B. H., *Exp. Parasit.*
2. Kupferberg, A. B., Johnson, G., Sprince, L., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 304.
3. Nuttall, G., *Z. f. Hyg.,* 1888, v4, 353.
4. Buchner, H., *Cent. f. Bakt.,* 1889, v5, 817; v6, 1 and 561.
5. Ehrlich, P., *Dtsch. Med. Wschr.,* 1898, v24, 597.
6. Pillemer, *et al., Science,* 1954, v120, 279.
7. Feldman, H. A., *Annals N. Y. Acad. Sci.,* 1956, v66, 263.

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Influence of Inorganic Ions on Fluoride Retention in the Rat.* (24087)

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Previous work has indicated that rats retain less fluoride in their carcass from a fluoride drinking water than from a chemically pure sodium fluoride solution when equal quantities of fluoride are given(1). These differences were thought to be due to mineral content of natural water, indicating that inorganic ions interfere with either fluoride absorption or skeletal retention. There is evidence for this assumption by Weddle and Muhler(2), and the theoretical studies of Feldman, Morken, and Hodge(3). The present study investigated several different inorganic ions, constituents of natural water supplies, for their influence on fluoride retention. Calcium, magnesium, iron, and phosphorus were chosen for investigation. These were evaluated for their ability to affect fluoride retention by comparing retention in the carcass of rats receiving fluoride from an aqueous solution containing the different inorganic ions with retention from a mineral-free water containing only fluoride.

* This work was supported in part by grant from Indiana State Board of Health.

† U.S.P.H.S. Pre-doctorate Fellow.

TABLE I. Results of Carcass Analyses of Rats Receiving Either a Pure Sodium Fluoride Solution (A) or One Supplemented with Calcium, Magnesium, Iron, and Phosphate (B).

Group	No. of animals	Avg ash wt (g)	μg F given	F found μg	F found ppm	Net % retained
A	9	5.258	731 2*	602 15*	116 3*	63.9 5.7*
B	9	5.466	732 1	503 14	92 2	49.1 5.7
Control	9	5.066		143 9	29 2	

* Stand. error.

Methods. The animals were weanling Sprague-Dawley strain rats. Littermates were used in all instances. The fluoride content of animals from each litter was determined at beginning of experiment so that fluoride retention during the experiment could be calculated. All fluoride solutions were made in advance for the entire study period, and stored in paraffined glass carboys. This insured that all groups received water of identical fluoride concentration. Carefully weighed amounts of the respective inorganic compounds were then added to the fluoride solutions. Concentrations of inorganic ions were decided upon by taking values of Indiana State Board of Health for representative cities in Indiana. The levels used were thus consistent with those found in natural communal sources. Calcium was studied at levels from 0 to 200 parts per million; magnesium from 0 to 160 parts per million; iron from 0 to 20 parts per million; and phosphorus from 0 to 80 parts per million. Fluoride concentrations were either 1 or 2 parts per million. All solutions were made using redistilled, mineral-free water. Calcium and magnesium were added as the chlorides, iron as ferrous sulfate, phosphorus as sodium dihydrogen phosphate, and fluoride as sodium fluoride. All chemicals were reagent grade. Animals were housed individually in raised screen cages in a temperature-controlled room. Water consumption was measured daily by recording the amount dispensed and spilled during drinking, using technics already described(1). Pair-drinking procedures assured uniform ingestion of fluoride water by all the animals. In all studies, a low-fluoride stock corn diet ($F = 0.5$ μg/g) was available *ad libitum*. At termination of an experimental period of either 4 or 6 weeks the animals were sacrificed and the whole carcasses analysed for fluoride by methods previously described (4).

Results. Table I summarizes the influence of a combination of Ca^{++}, Mg^{++}, Fe^{++}, and PO_4^{---} ions on the whole carcass retention of fluoride. Group A received redistilled mineral-free water containing 1 μg F/ml. Group B received the same amount of fluoride as Group A, but the water contained in addition 200 parts per million calcium, 160 parts per million magnesium, 20 parts per million iron, and 80 parts per million phosphate. These data show an appreciable reduction in total amount of fluoride retained by the Group B animals when compared to those of Group A, showing that the combination of ions resulted in significant ($p = 0.02$) inhibition of fluoride retention.

The results of the studies in which only calcium was aded to the fluoride drinking water are summarized in Table II. No significant reduction in retention occurred as increasing

TABLE II. Results of Whole Carcass Analyses of Rats Receiving Fluoride and Varying Levels of Calcium in Drinking Water.

Group	No. of animals	ppm Ca	μg F given	F found μg	F found ppm	Net % retained
A	10	0	584 10*	516 29*	76 5*	62.0 4.2*
B	4	8	559 9	524 72	75 5	60.6 6.9
C	4	16	567 10	496 16	80 0	55.4 4.1
D	16	22	568 9	496 21	71 4	62.4 4.4
E	11	107	568 6	481 13	64 3	64.3 2.1
F	12	197	596 7	454 14	60 3	55.6 3.9

* Stand. error.

TABLE III. Results of Whole Carcass Analyses of Rats Receiving Fluoride and Varying Levels of Magnesium in Drinking Water.

Group	No. of animals	ppm Mg	μg F given		F found μg		F found ppm		Net % retained	
A	15	0	545	5*	505	14*	73	2*	71.4	3.1*
B	2	10	524	7	483	16	61	11	58.6	2.2
C	2	20	529	3	508	7	75	5	62.8	1.7
D	6	40	531	5	487	20	70	20	74.1	5.2
E	5	80	544	8	474	25	73	4	71.6	6.6
F	2	160	560	2	524	50	70	1	85.2	8.7

* Stand. error.

TABLE IV. Results of Whole Carcass Analyses of Rats Receiving Fluoride and Varying Levels of Iron in Drinking Water.

Group	No. of animals	ppm Fe	μg F given		F found μg		F found ppm		Net % retained	
A	8	0	1103	7*	640	40*	96	5*	54.9	3.8*
B	8	5	1104	7	673	50	95	6	57.5	4.6
C	7	10	1101	7	709	25	98	5	60.7	3.2
D	7	20	1105	8	633	41	96	8	54.0	3.3

* Stand. error.

levels of calcium were added to the drinking water.

The influence of magnesium ions on availability of fluoride for whole carcass retention is found in Table III. There were no significant differences in any of the groups in amount or fluoride given, total amount recovered in the carcass, or in concentration of fluoride in the carcass.

The results of the effect of iron appear in Table IV. Iron at levels of 5 to 20 ppm did not influence availability of fluoride for whole carcass retention.

The effect of phosphate is reported in Table V. These data suggest that phosphate, like magnesium, appeared to facilitate somewhat the storage of fluoride although not in proportion to phosphate concentration studied. Certainly no reduction in storage occurred with addition of phosphate to the water.

Discussion. Considerable attention has been given in the past to factors affecting fluoride metabolism and, especially, fluoride retention in the body when fluoride is used in communal water supplies as a means of partially reducing incidence of dental caries in humans. Present evidence suggests that when fluorides are added to fluoride-deficient water supplies at the level of 1 μg/ml there are no adverse systemic effects occurring from cumulative fluoride retention.

The degree of dental caries reduction afforded by artificial addition of fluoride to communal water supplies containing different interfering inorganic ions is of particular interest, however. The results of such interference may be an inactivation of the metabolically available fluoride to such an extent that degree of protection against dental caries is significantly affected. Because of this possibility, the inorganic composition of the communal water supplies which are being fluoridated may need more attention than given heretofore in order to insure availability of sufficient fluoride to obtain maximum dental caries reduction. It may be that amount of

TABLE V. Results of Carcass Analyses of Rats Receiving Fluoride and Varying Levels of Phosphate in Drinking Water.

Group	No. of animals	ppm PO_4	μg F given		F found μg		F found ppm		Net % retained	
A	9	0	1149	6*	791	22*	106	3*	43.3	2.1*
B	9	20	1129	9	841	28	107	5	48.7	2.8
C	8	40	1151	4	799	21	100	3	44.0	1.8
D	9	80	1141	5	827	25	105	4	46.7	2.1

* Stand. error.

fluoride added to communal water supplies will need to be increased in communities served by water having a high inorganic ion content in order to maintain significant anticariogenic benefits. The data in this study apply to this problem, since they strongly suggest that when fluoride is added to a water supply containing Ca^{++}, Mg^{++}, Fe^{++}, and PO_4^{---} together, significantly less fluoride is retained in the whole rat carcass than when similar amounts of fluoride are added to a mineral-deficient water. The contribution of individual inorganic ions in producing this collective effect is not clearly defined, since when Ca^{++}, Mg^{++}, Fe^{++}, or PO_4^{---} ions are tested separately by measuring fluoride retention in the whole carcass, no significant changes are noted. As a result, additional study is needed of combinations of ions common to communal water supplies to determine the unique properties of the ion combination which make it capable of significantly affecting fluoride absorption or retention.

Summary. 1) The whole carcass retention of fluoride by rats receiving a fluoride drinking water containing a combination of Ca^{++}, Mg^{++}, Fe^{++}, and PO_4^{---} ions was significantly less than in rats drinking a chemically pure fluoride water containing no additional inorganic ions. 2) In contrast, studies of the individual ions at various concentrations show only small fluctuations in the proportion retained. No significant influence on retention was noted when any of the ions were studied singly. 3) The decrease in retention seen in rats ingesting the water containing a combination of ions suggests that the influence of the ions may be additive or synergistic. This may be important since most natural waters contain a group of several inorganic constituents. More attention should be placed on inorganic composition of the communal water supplies before fortifying them with fluoride. It is possible that if the combination of major inorganic constituents of natural waters exceeds a yet-to-be determined concentration, optimal fluoride concentration would need to differ from the now accepted level of 1 ppm in order to produce maximum anticariogenic benefits.

1. Wagner, M. J., Muhler, J. C., *J. Dent. Res.*, 1957, v36, 552.

2. Weddle, D. A., Muhler, J. C., *J. Nutrition,* 1954, v54, 437.

3. Feldman, J., Morken, D., Hodge, H. C., *J. Dent. Res.,* 1957, v36, 192.

4. Weddle, D. A., Muhler, J. C., *ibid.,* 1955, v34, 900.

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Effect of Banana Ingestion on Urinary Excretion of 5-Hydroxyindole Compounds in Normal Adults. (24088)

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Anderson and coworkers(1) have reported that addition of bananas to the diet increases the urinary excretion of 5-hydroxyindole acetic acid (5-HIAA). Their study was done on 4 Rhesus monkeys and 2 sick children. 5-hydroxyindole (5-OH indole) compounds are excreted in larger than normal amounts in the clinical syndrome produced by malignant carcinoid with metastases, and both the roughly quantitative "screening test"(2,3), and the precise quantitative determination(4) are useful laboratory procedures in establishing the diagnosis. Anderson's cited report suggests that high levels of urinary 5-HIAA may be produced in normal patients by banana ingestion, thus leading to an erroneous diagnosis of malignant carcinoid.

The present investigation was undertaken to determine whether urinary excretion of 5-OH indole compounds in normal adults can be affected significantly by addition of bananas to the diet.

Method. Measurements were made on urine from ten normal adults, 7 males and 3 females. For 5 days prior to collection of control 24-hour urine specimens the subjects ate their usual diets, avoiding bananas or medication. Subsequently, each individual added to his normal diet 12 g of peeled banana per kg of body weight over a period of 24 hours, an amount equal to or in excess of that used by Anderson *et al.* in the children. Simultaneously, 24-hour urine specimens, preserved with toluene, were collected for 5-OH indole determinations. A simple screening test(2) and a quantitative test(3) were used in these determinations.

Results. The data are presented in Table I. Banana ingestion (12 g/kg per 24 hr) by normal adults produced an average 2-fold (statistically significant : $T = 12.88$; $P < 0.1\%$) increase in urinary excretion of 5-OH indoles. There was no significant difference between males and females. In no case did total excretion fall within the range that might be interpreted as indicating the presence of malignant carcinoid.

Results with the simple screening procedure (2), however, could have led to erroneous interpretations. On 3 occasions the screening test was weakly positive or moderately positive. Interpreting these tests in accordance with the criteria of Sjoerdsma *et al.*(2), one would have to assume that excretion of 5-HIAA in the urine of these 2 normal subjects was at least 40 mg per 24 hours. Quantitative determinations showed that when total 24-hour urinary output was considered, total excretion of 5-OH indole compounds fell below 40 mg per 24 hours (Table I).

Discussion. Banana ingestion in normal adults increases the 24-hour urinary excretion of 5-OH indole compounds. The increase observed, a 2-fold one, is of such magnitude that an erroneous diagnosis of malignant carcinoid would be unlikely. These findings are in contrast to Anderson's *et al.* using monkeys and children(1). These authors found a 5 to 30-fold increase in the amount of 5-HIAA excreted in Rhesus monkeys, and an average 8.7-fold increase in the children after banana feeding.

TABLE I. Effect of Banana Ingestion on Urinary Excretion of 5-OH Indole Compounds in Normal Adults.

			Urinary 5-OH indoles (mg/24 hr)		
Age, yr	Wt, kg	Sex	Base-line diet (C)	Banana added (B)*	Ratio B/C
42	68.1	♂	25.0	36.6	1.46
36	77.2	♂	19.0	30.5	1.60
31	63.6	♂	11.9	27.2	2.28
30	68.1	♂	16.1	33.2	2.06
34	80.9	♂	12.3	33.5	2.72
36	86.3	♂	12.7	22.6	1.77
35	77.2	♂	14.7	29.8	2.02
25	56.8	♀	12.1	25.5	2.10
25	56.8	♀	13.0	31.5	2.42
28	54.5	♀	15.0	26.8	1.79
		Total	151.8	297.2	20.22
Avg					
32.4	69.0		15.2	29.7	2.02

* 12 g peeled bananas/kg body wt.
$T = 12.88$; $P < 0.1\%$.

Species differences may account for the noted discrepancy in urinary 5-OH indole excretion between humans and Rhesus monkeys following ingestion of banana. General diet differences, variation in states of health, and obvious differences in maturity are factors that must be taken into account in explaining the disparity of results between children and adults. It seems apparent that findings in Rhesus monkeys and in children in relation to metabolism of 5-OH indole compounds cannot be directly extrapolated to adult humans.

Routine screening determinations of 5-OH indole compounds based on a single urine specimen may give a false positive result. Quantitative examination of these 5-OH indole compounds based on a 24-hour urine specimen is a more valid evaluation. An initial positive screening test when quantitated may fall within the normal range of values when corrected for 24-hour urinary output.

It must be remembered that the quantitative test used in this experiment determines total 5-OH indoles, of which 5-HIAA comprises about 75%(5).

This phenomenon of increased urinary excretion of 5-HIAA(1) in monkeys and sick children following addition of banana to the diet prompted Waalkes *et al.*(6) to analyze bananas for physiologically active substances. They found that bananas contain large

amounts of serotonin, norepinephrine and dopamine. This finding suggests that dietary (exogenous) precursors can increase the urinary excretion of 5-OH indole compounds.

Summary. 1. Banana ingestion (12 g/kg/24 hr) by normal adults produced an average 2-fold increase in urinary excretion of 5-OH indoles. This was not of such magnitude as to suggest an erroneous diagnosis of malignant carcinoid. 2. Collection of 24-hour urine specimens and use of a quantitative test are advocated over collection of random urine specimens. Positive screening tests should be checked by a quantitative method.

1. Anderson, J. A., Ziegler, M. R., Doeden, D., *Science,* 1958, v127, 236.

2. Sjoerdsma, A., Weissbach, H., Udenfriend, S., *J.A.M.A.,* 1955, v159, 397.

3. Ross, G., Weinstein, I. B., Kabakon, B., *Clin. Chem.,* 1958, v4, 66.

4. Udenfriend, S., Titus, E., Weissbach, H., *J. Biol. Chem.,* 1955, v216, 499.

5. Sjoerdsma, A., Weissbach, H., Udenfriend, S., *Am. J. Med.,* 1956, v20, 520.

6. Waalkes, T. P., Sjoerdsma, A., Creveling, C. R., Weissbach, H., Udenfriend, S., *Science,* 1958, v127, 648.

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Hemagglutination with Adenoviruses. Nature of Viral Antigen. (24089)

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It has been demonstrated that antigens can be adsorbed to tannic acid-treated erythrocytes so that the red blood cells agglutinate when mixed with specific antiserum(1,2,3). An attempt therefore was made to apply this technic to a study of the antigens of adenoviruses and the antibodies directed against these viral materials. Since the completion of this investigation it has been reported that antibodies directed against adenoviruses(4), herpes simplex(5), and poliomyelitis(6) viruses can be measured by use of tannic acid-treated sheep erythrocytes. The study to be reported confirms the data presented by Friedman and Bennett(4), and indicates that a modified hemagglutination technic can measure not only the adenovirus group antigen but also type-specific antibodies in approximately 50% of the sera tested. In addition, identification of the major antigen which adsorbs to tannic acid-treated erythrocytes will be described.

Materials and methods. Tissue culture. Strain HeLa cells (Gey) derived from an epidermoid carcinoma of the uterus were employed. The methods used for serial propagation and preparation of tube cultures have been described(7). The nutrient fluid consisted of 40% human serum and 60% Hanks' balanced salt solution or Eagle's basal medium supplemented with 10% human serum. *Viruses.* Adenoviruses types 1, 2, 3 and 4 were grown in HeLa cells using a maintenance mixture consisting of Scherer's amino acid-vitamin mixture (MS) 67.5%, chicken serum 7.5%, and tryptose phosphate broth 25% (8). Virus suspensions were prepared by freezing and thawing infected cells 6 times following which cellular debris was removed by centrifugation(7). Viruses were stored in sealed glass ampules at –70°C or in screw cap tubes at –30°C. *Hemagglutination (HA) test.* A modification of the method reported by Stavitsky(3) was used. Chicken erythrocytes were employed because a more distinct endpoint was obtained with these cells than with sheep or rabbit red blood cells. Erythrocytes from white leghorns were obtained weekly and stored in Alsever's solution at 4°C. Before use, cells were washed 3 times with

* Work carried out in partial fulfillment of requirements of Project Teaching Program of Medical Curriculum, Western Reserve University, School of Medicine.

0.85% NaCl buffered at pH 7.1 with 0.15 M phosphate. This salt solution will henceforth be called saline. A 2.5% suspension of cells was prepared and standardized at a reading of 500 with a 540 mμ filter in a Klett colorimeter. The 2.5% suspension of chicken rbc was incubated with an equal volume of a 1:20,000 solution of tannic acid at 37° for 10 minutes. The treated cells were washed once with an equal volume of saline and then resuspended to 2.5% in saline. Tanned rbc were prepared daily. Virus suspension prepared as described was diluted with an equal volume of saline in order to obtain results compatible with both economy and antibody titers high enough to be interpreted. Adsorption of viral antigen to tanned rbc ("sensitization") was carried out by mixing 1 volume of cells, 1 volume of diluted virus suspension and 4 volumes of 0.15 M phosphate buffer at pH 6.4. Tanned rbc and virus were incubated at room temperature for 10 minutes. Sensitized cells were centrifuged at 1000 rpm for 5 minutes, washed twice with a 1:100 normal rabbit serum solution in saline, and resuspended in 1:100 normal rabbit serum as a 1% suspension. Equal volumes (0.3cc) of the suspension of virus-treated cells and serial dilutions of serum were incubated at room temperature for exactly 60 minutes and read for hemagglutination. The last tube in which definite hemagglutination was observed was considered as the endpoint. All titers are expressed as the reciprocal of the final dilution of serum. *Sera.* Acute and convalescent human sera from cases of proved adenovirus infections were centrifuged free of particulate matter and heated at 56°C for 30 minutes. Serial 2-fold serum dilutions were prepared in 1:100 normal rabbit serum. *Serum neutralization and virus infectivity titrations* were done exactly as described previously(7). A standard *complement-fixation* titration using 2 units of complement was employed. The highest dilution of serum which caused complete fixation of complement was taken as the endpoint. The titer is expressed as the reciprocal of the final dilution of serum in the initial phase of the reaction.

Results. The technic for adsorption of antigen to tannic acid-treated red blood cells was shown to be applicable to adenoviruses. The major modification in the technic described by Stavitsky(3) was the use of chicken erythrocytes to obtain sharp and reproducible endpoints readily and after a short period of incubation (60 minutes). Results of experiments which investigated conditions for sensitization of red blood cells with adenovirus antigen, heat stability of the antigen, and quantity of antigen necessary to obtain maximum hemagglutination confirmed the studies reported by Friedman and Bennett (4) and therefore will not be described here. Moreover, as their report indicates, it demonstrated an increase in titer of antibodies in sera of patients convalescent from adenovirus infections. By technics employed in the initial part of this study and by previous workers, however, antibody titer in the acute phase serum was occasionally high, and an antibody rise could be detected with 2 or more types of virus. That is, the antibody demonstrated by the hemagglutination method, just as with complement-fixation titration, appeared to be group rather than type specific.

Experiments were therefore designed to develop a method by which type-specific antibodies could be measured, on the assumption that, if the heterologous viruses were pooled and used as "blocking antigens" to react with non-type-specific antibodies, the type-specific antibody could be detected. The "blocking antigen" pool consisted of equal volumes of each of the 3 heterologous viruses. To 0.15 cc of each serial dilution of the serum to be tested was added 0.15 cc of the "antigen pool." The serum dilution-virus pool mixtures were incubated at 37°C for 30 minutes after which there was added to each tube 0.3 cc of a suspension of tanned rbc previously reacted with the type of virus for which antibodies were to be measured. The results of an experiment with and without the use of "blocking antigens" are summarized in Table I.

By neutralization titrations the sera being tested showed a rise in type 3 antibodies, a questionable rise in type 2 antibodies, and no increase in type-specific antibodies to type 1 or 4 virus. Without the use of the pools of

TABLE I. Effect of Heterologous Virus Pools as "Blocking Antigens" on Hemagglutination Antibody Titers.

Antigen		HA antibody titer of serum			
		No "blocking antigens"		With "blocking antigens"	
Serum mixed with virus pool	Virus adsorbed to tanned RBC	Acute	Conval.	Acute	Conval.
Types 2, 3, 4	Type 1	>4096	>4096	>5120	>5120
1, 3, 4	2	4096	"	< 20	20
1, 2, 4	3	2048	4096	"	>5120
1, 2, 3	4	"	"	1280	1280
—	Normal HeLa	<8	<8	<8	<8

heterologous viruses it was not possible to determine the specific type of adenovirus responsible for the infection. With "blocking" viral antigens a clear cut rise in type 3 antibody was measured as well as a slight increase in type 2 antibodies. These results were similar to those obtained by neutralization titrations. Although results were not always as definitive as in this experiment, the data implied that pre-incubation with "blocking antigens" was a useful procedure in order to detect a type-specific reaction.

To determine the specificity of the hemagglutination reaction, the results of hemagglutination, neutralization, and complement-fixation titrations with the same sera were compared. To quantitate antibodies by the hemagglutination technic, "blocking" heterologous viruses were employed. Eight pairs of sera were tested. In 4 pairs the antibody responses to infection as measured by hemagglutination were identical to those obtained with neutralization. In the remaining 4 pairs of sera, results of hemagglutination titrations were similar to those demonstrated with the complement-fixation technic in that the specific type of virus which caused infection could not be determined; rather an increase in antibody titer for 2 or more types was observed. The reasons for the inability of blocking antigens to eliminate non-specific reactions regularly have not been ascertained. Results of antibody titrations with 2 pairs of sera, selected to illustrate range of variation noted, are summarized in Table II. A type-specific rise in hemagglutination antibodies in serum pair II closely mimics the results obtained by neutralization titration. It is interesting to note that although there is a high titer of type 1 antibodies in the acute phase serum, the specific etiology of the infection could be demonstrated.

Identification of viral antigen adsorbed to tanned rbc. To determine whether the antigenic material which adsorbed to chicken erythrocytes was part of the viral particle *per se* or was a smaller antigenic particle, the viral suspensions were centrifuged at 105,400 x g for 60 minutes in a Spinco Model L preparative centrifuge. The upper one-half of the supernate was removed carefully, the remaining fluid discarded, and the sediment resuspended to the original volume in Hanks' balanced salt solution. The results of infectivity titrations and hemagglutination antibody titrations done with the original viral suspensions, supernates, and resuspended sediments of the type 1 and 3 viruses are summarized in Table III. The supernatant fluids contained less than 1% of the infectious virus in the uncentrifuged material and the sediment comprised 10 to 32% of the total infectious virus. Despite the relatively small amount of infectious agent remaining in the supernate, the hemagglutination antibody titer measured by rbc sensitized with this fraction was similar to that obtained with rbc mixed with the original viral suspension. Erythrocytes treated with the resuspended sediment, however, yielded considerably lower antibody titers. The results reported by Friedman and Bennett(4) and here confirmed indicated that antibody titer obtained by hemagglutination titration was directly proportional to quantity of virus employed to sensitize rbc. These quantitative considerations indicate that each supernate used in the experiments described in Table III contained

TABLE II. Comparison of Antibody Titers as Measured by Hemagglutination, Complement-Fixation and Neutralization Titrations.

Serum	Antibody titration method	Antibody titer — Type virus 1	2	3	4	HeLa control
I acute *	Hemagglutination	32	2048	8	8	16
conval.		2048	"	512	512	"
I acute	Neutralization	<8	<8	8	<8	
conval.		"	8	512	8	
I acute	Complement-fixation	"	8	<8	<8	
conval.		128	256	128	128	
II acute	Hemagglutination	4096	32	32	32	16
conval.		"	"	512	"	"
II acute	Neutralization	<8	<8	<8	<8	
conval.		"	"	32	"	
II acute	Complement-fixation	16	8	<8	"	
conval.		128	256	256	256	

* Acute and convalescent phase.

as much antigenic mass adsorbable onto tanned rbc as the uncentrifuged specimen, and significantly more rbc-adsorbable antigen than was present in the sediment fraction although the latter material had 10 to 30 times more infectious virus. Similar results were obtained in experiments carried out with types 2 and 4 adenoviruses. These data clearly imply that the major antigen responsible for the hemagglutination of rbc when mixed with specific antibody is viral material which is separable from the infectious viral particle and probably smaller in size than the infectious unit.

Discussion. Adsorption of antigens to tannic acid-treated erythrocytes, first demonstrated with bacterial products(1), has been applied to adenoviruses(4), herpes simplex (5), and poliomyelitis(6) viruses. Agglutination of the treated and sensitized rbc by specific antibodies is the indicator by which antigen is detected and also serves as a technic for quantitation of antibodies. The evidence here presented confirms previous reports(4) that this technic does not measure adenovirus type-specific antibody. Rather antibodies to the group antigen, as detected by complement-fixation test, are titrated by the hemagglutination technic. A preliminary reaction of serum with a pool of heterologous viruses, however, permitted utilization of the hemagglutination technic to measure type-specific antibodies in 4 of 8 sera. It may be possible to obtain type-specific antibody rises in more sera if the pool of heterologous antigens contains more of the known types of adenoviruses. The data imply that antibody detected by hemagglutination titration is directed chiefly against an antigen of the homologous virus. Although comparable experiments have not been done by complement-fixation titrations, some evidence suggests a similar antigen may be involved in this technic(9).

TABLE III. Effect of High Speed Centrifugation* on the Hemagglutination Antigen and the Infectious Virus.

Virus type	Material tested	Infectivity titer (log)	HA antibody titer†
1	Original	−5.0	128
	Supernate	−2.5	128
	Sediment	−4.0	16
3	Original	−3.0	512
	Supernate	−1.0	1024
	Sediment	−2.5	16

* Viral suspensions centrifuged at 105,400 × g for 60 min.

† Dilutions of a human convalescent serum were mixed with tanned red blood cells previously treated with the viral material to be tested.

High speed centrifugation of adenovirus-infected culture fluids demonstrated that the antigen which adsorbs to tanned chicken rbc is separable from the viral particle and is soluble. This evidence further suggests that the antigen may be similar to that responsible for complement-fixation. The hemagglutination technic offers not only another means of measuring viral antibodies, but also intro-

duces a sensitive method for detecting small quantities of viral antigens and for studying differences in antigens related to a single virus.

Summary. A rise in antibodies to types 1-4 of adenoviruses during illness can be detected in human serum by employing hemagglutination technics. Although the original method described does not measure an increase in antibodies directed against a single type of virus, type-specific antibodies can be titrated if serum is mixed with a pool of heterologous viruses before tanned rbc treated with homologous virus are added. This modification measured type-specific antibodies solely in 4 of the 8 sera tested. The antigen adsorbed to tannic acid-treated rbc is probably not the infectious viral particle and is separable from it by high speed centrifugation.

1. Boyden, S. V., *J. Exp. Med.*, 1951, v93, 107.
2. Fisher, S., *Am. J. Hyg.*, 1952, v50, 445.
3. Stavitsky, A., *J. Immunol.*, 1954, v72, 360.
4. Friedman, M., Bennett, C. R., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 712.
5. Scott, L. V., Felton, F. G., Barney, J. A., *J. Immunol.*, 1957, v78, 211.
6. McKenna, J. M., Zuschek, F., Frankel, J. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 160.
7. Ginsberg, H. S., Badger, G. F., Dingle, J. H., Jordan, W. S., Jr., Katz, S., *J. Clin. Invest.*, 1955, v34, 820.
8. Ginsberg, H. S., Gold, E., Jordan, W. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 66.
9. Pereira, H. G., *J. Path. and Bact.*, 1956, v72, 105.

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Variation and Hemolysin Production in Relation to Virulence of *Leptospira pomona.** (24090)

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Properties of leptospirae which contribute to their virulence have not been well established. Fukushima and Hosoya(1) and Higuchi(2) demonstrated substances toxic for guinea pigs in cultures of *Leptospira icterohemorrhagiae* maintained anaerobically or *in vacuo*. Stavitsky(3) was unable to verify these findings. Fibrinolysin, leukocidin, and coagulase could not be demonstrated in filtrates of leptospiral cultures(4). Volland and Brede reported(17) that hyaluronidase was produced by leptospirae *in vitro*. A soluble hemolysin has been demonstrated in cultures(5,6) and it has been suggested that the hemolytic activity of leptospirae is an attribute of a toxin(5). Pathogenic leptospirae decrease in virulence upon continual transfer in culture media(8). Presumably reduction in virulence of a given culture is due to *in vitro* development of avirulent variants. The present investigations concern(1) changes in virulence of *Leptospira pomona* for hamsters following passage in media, and (2) *in vitro* hemolysin production as correlated to virulence.

Materials and methods. Virulence determinations. Two strains of *L. pomona* were employed. Strain W had been isolated from bovine urine(9) and maintained by serial passage in weanling guinea pigs. Although this strain was not lethal for hamsters, it was pathogenic for cattle, swine, sheep, goats, and dogs. Strain O had been maintained in continuous hamster and guinea pig passage since its isolation from porcine urine. Strain O killed hamsters and was pathogenic for sheep, swine, and dogs. The first medium passage of Strain W was obtained as blood culture from a sheep which had been inoculated with infected guinea pig blood. Medium passages of Strain O were initiated by culturing blood from an infected guinea pig. Maximum growth of initial hemocultures was obtained after approximately 30 days. Chang's fluid medium(10),

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containing final concentration of 10% sterile rabbit serum and 0.01% hemoglobin (Difco), was used for cultivation. The medium was dispensed in approximately 10 ml amounts in screw cap culture tubes. Inocula consisted of 0.1 to 0.5 ml of culture from previous medium passage. Cultures were incubated at 29°C. Medium passages and virulence titrations in animals were made when cultures were 5 to 10 days old. At this time growth was optimal and the number of leptospirae was ascertained in a Petroff-Hauser bacterial counting chamber using darkfield illumination at 420x. Inocula for animals were prepared using a diluent which consisted of basic ingredients of Chang's fluid medium(10). Tenfold serial dilutions were made and each animal was inoculated intraperitoneally with 1.0 cc of the respective dilution. Both 3 to 5-week hamsters and 4 to 6-week old guinea pigs were from disease free colonies and raised by the authors. Five hamsters were employed/dilution except in the first medium passage titration of Strain W, in which 7 hamsters were used. In guinea pig titrations 5 animals were employed/dilution and were conducted for medium passages 1, 4, and 8 of Strain W and passages 4 and 11 of Strain O. Hamsters were observed for signs of illness. Blood was obtained from moribund hamsters by cardiac puncture using sterile 2 cc syringe wetted with heparin. The plasma was separated and examined by darkfield microscopy for presence of leptospirae. Concentration of organisms in the plasma was determined using counting chamber method. Animals which survived were sacrificed 20 to 30 days after inoculation and their sera examined for leptospiral antibodies using a modified agglutination-lysis test(11). Virulence of each culture was expressed as number of leptospirae which would kill 50% of inoculated hamsters (LD_{50}). LD_{50} values were calculated by the method of Reed and Muench(12). *Hemolysin determinations.* Cultures of *L. pomona* of various degrees of virulence were inoculated into isotonic buffered medium described by Alexander, *et al.*(5) supplemented with 0.01% hemoglobin (Difco). The cultures were incubated at 29°C for 10 to 14 days, when approximately 10^8 leptospirae/cc were present. Growth phase of cultures was that at which optimum hemolysin production has been reported to occur(5). Following incubation, cultures were stored in refrigerator (4°C). Sheep, cow, hamster, rabbit, and guinea pig erythrocytes were obtained and preserved in sterile Alsever's solution(13). Erythrocytes were washed 3 times in isotonic buffer(5) and resuspended to make a 5% cell suspension. A portion of rbc suspension was used to make standard solutions of 10 to 100% hemolysis. Procedure for titration of hemolysin has been reported by Russell(6). Following incubation, the tubes were gently agitated and centrifuged at 1500 rpm for 10 minutes. A 1 ml amount of supernatant fluid from each tube was diluted with 2 ml of isotonic buffered base to make a total volume of 3 ml. Optical density of hemolysis standards and diluted supernatant fluid was determined in Bausch and Lomb Spectronic 20 colorimeter at 520 mμ. Percent hemolysis was ascertained from standard curve of % hemolysis *vs.* optical density. Hemolytic activity of cultures was expressed as that dilution of culture which produced 50% hemolysis of a 5% washed rbc suspension.

Results. Virulence determinations. Virulence of Strain W for hamsters was determined for medium passages 1, 4, 8, 10, 12, 16, 20, and 24. The number of leptospirae required to infect 50% of hamsters inoculated with the first medium passage of nonlethal W strain was calculated to be 3.5. While death of hamsters did not occur at any dilution of the first medium passage, there were deaths in all subsequent titrations of this strain (medium passages 4-24). Thus, an increase rather than a decrease in virulence occurred during passage. Surviving animals did not have demonstrable serum antibodies. An examination of plasma from moribund animals revealed 10^7 to 10^8 leptospirae/cc. Hamsters also died when inoculated with blood from moribund animals or with cultures of this blood. Cell free culture filtrates of Strain W did not produce death of hamsters following intraperitoneal inoculation.

Virulence of Strain W decreased from pas-

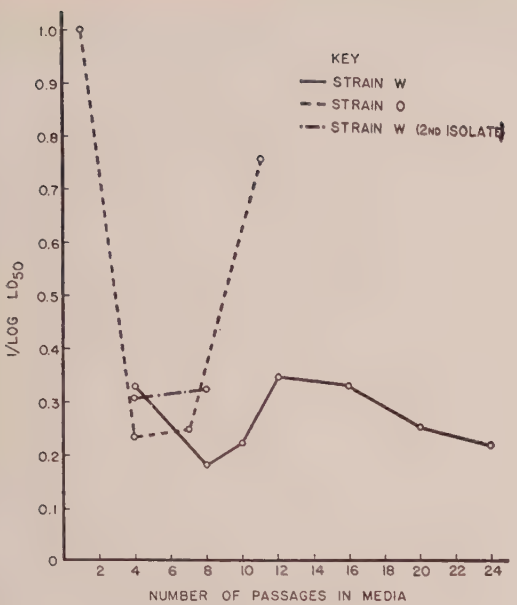

FIG. 1. Effect of passage in media on virulence of *L. pomona* for hamsters.

sage 4 to 8. An increase in virulence was noted for passages 10 and 12 after which virulence decreased again. Lethality for hamsters was again demonstrated for Strain W after 4 medium passages of a culture obtained from another experimentally infected sheep. Virulence determinations with passages 1, 4, 7, and 11 of Strain O indicated an initial diminution of virulence followed by an increase. The LD_{50} of the first passage was approximately 1. The results are summarized in Fig. 1.

Comparison of survival time of infected hamsters and size of inoculum was made. A linear relationship occurred with both strains. The *in vivo* generation time, calculated according to the method of Youmans and Youmans(14), was 7.5 hrs. Cultures of low virulence showed a similar generation time but average survival time was delayed as compared to highly virulent cultures. These findings are in agreement with previous reports employing *L. icterohemorrhagiae* in guinea pigs(15), where the generation time was 8.3 hours.

Hamsters and guinea pigs were equally susceptible to infection with either strain of *L. pomona*.

Hemolysin determinations. A direct relationship between *in vitro* hemolytic activity of *L. pomona* and virulence was not observed. Table I shows that the greatest hemolysin production was associated with a culture of Strain W which had a relatively low degree of virulence ($LD_{50} = 5x10^5$). Cultures which were not lethal for hamsters did not consistently produce lesser amounts of hemolysin. Recent observations (unpublished) have indicated that the hamster lethal variant of Strain W produced a significant *in vivo* hemolytic effect. Marked *in vivo* hemolysis was not observed for Strain O. A consistent difference in hemolysin production *in vitro* for Strains W and O was not evident. Sheep and cow erythrocytes were more susceptible to *L. pomona* hemolysin than were those of rabbits and hamsters. Hemolytic activity was not demonstrated with guinea pig rbc. Relative hemolytic activity of the cultures was not affected by the different rbc types employed.

Discussion. The change from non-lethality to lethality of Strain W for hamsters following cultivation in media is paradoxical but not an isolated incident in evolvement of greater virulence for the genus. Van Riel has been cited(5,7) to have observed an increase in pathogenicity for guinea pigs of *L. icterohemorrhagiae* following 8 months of *in vitro* cultivation. Residence of Strain W in sheep may have enhanced the opportunity for mutation to occur.

The decrease in LD_{50} values (virulence in-

TABLE I. *In Vitro* Hemolytic Activity of *L. pomona* for Sheep Erythrocytes in Relation to Virulence.

Strain	History	LD_{50}	Units of hemolysin
W	Semi-annual transfers in semi-solid medium, 3 yr	Not lethal*	17
"	3 transfers in media following isolation from guinea pig	*Idem*	48
"	4 transfers in media following isolation from sheep	1.5×10^3	80
"	10 transfers *Idem*	1.0×10^3	30
"	25 transfers "	5.0×10^5	230
O	4 transfers in media following isolation from guinea pig	1.5×10^4	57
"	8 transfers *Idem*	3.3×10^3	31

* Culture not lethal for hamsters.

crease) observed with both W and O strains following 4 to 8 passages in media may be explained on the basis of a reverse mutation. The initial decrease in virulence (medium passages 1-4,8) would be due to development of avirulent variants. Subsequently (medium passages 4-11 of Strain O and 8-12 of Strain W) a mutation resulted in production of virulent organisms capable of outgrowing the parent cells *in vitro*. A mutation back to the slower growing virulent cells would account for virulence decrease observed with passages 12 to 24 of Strain W. Data pertaining to variation among leptospirae are meager due to lack of suitable and precise technics. Unfortunately, Cox's solid leptospiral medium(16) did not support growth of either Strain W or O.

Correlation of *in vitro* hemolytic activity and degree of virulence was lacking. The leptospiral hemolysin may contribute some aggressive action *in vivo*, or there may be several hemolytic factors involved with separate systems operating *in vivo* and *in vitro*. It is doubted that the pathogenicity of *L. pomona* is exclusively due to hemolysin, although hemolytic anemia and its cellular ramifications may be the prominent syndrome in severe, acute leptospirosis. The *in vivo* release of hemoglobin is probably beneficial in some degree to leptospirae since hemoglobin or hemoglobin-like compounds are definitely growth stimulative *in vitro*. Probably an endotoxin, entirely independent of the hemolysin, enables the microorganisms to cause damage to tissues and persist in the host. Imamura, *et al.*(18) have recently reported that a toxic product and a hemolysin produced by *L. icterohemorrhagiae* could be distinguished on the basis of thermal stability.

Summary. 1) An investigation was made of changes in virulence of *L. pomona* for hamsters following serial transfers in a fluid medium. *L. pomona* strain W, which did not initially kill hamsters, later produced a lethal effect. Both strain W and O of *L. pomona* decreased in virulence (increase in LD_{50} values) during first 4 medium passages. After 4 to 8 passages in media an increase in virulence was observed. Titrations of subsequent medium passages (12 to 24) of strain W indicated a gradual loss of virulence. 2) *In vitro* hemolysin production could not be correlated with virulence. An explanation for virulence alterations is proposed and the possible significance of hemolytic activity of leptospirae is discussed.

1. Fukushima, B., Hosoya, S., *Sci. Rep. Govt. Inst. Infect. Dis., Tokyo Imp. Univ.*, 1926, v5, 151.

2. Higuchi, S., *Fukuoka Acta Med.*, 1931, v24, 3.

3. Stavitsky, A. B., *J. Inf. Dis.*, 1945, v76, 179.

4. ———, *Bact. Rev.*, 1948, v12, 203.

5. Alexander, A. D., Smith, O. H., Hiatt, C. W., Gleiser, A. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 205.

6. Russell, C. M., *J. Immunol.*, 1956, v77, 405.

7. Van Thiel, P. H., *The Leptospiroses*, Universitae Pers Leiden, Leiden, 1948.

8. Wolff, J. W., *The Laboratory Diagnosis of Leptospirosis*, Charles C. Thomas, Springfield, Ill., 1954.

9. Morse, E. V., Allen, V., Krohn, A. F., Hall, R., *J.A.V.M.A.*, 1955, v127, 417.

10. Chang, S. L., *J. Inf. Dis.*, 1947, v81, 28.

11. Morse, E. V., Allen, V., Pope, E. P., Krohn, A. F., *J.A.V.M.A.*, 1955, v127, 422.

12. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 439.

13. Mayer, M. M., Osler, A. G., Bier, O. G., Heidelberger, M., *J. Exp. Med.*, 1946, v84, 535.

14. Youmans, G. P., Youmans, A. S., *Am. Rev. Tuberc.*, 1951, v64, 534.

15. Faine, S., *Brit. J. Exp. Path.*, 1957, v38, 8.

16. Cox, C. D., Larson, A. D., *J. Bact.*, 1957, v67, 619.

17. Volland, W., Brede, H. D., *Med. Monatsschr.*, 1951, 698. Reviewed by Broom, J. C., *Bull. Hyg.*, 1952, v27, 251.

18. Imamura, S., Kyunosuke, K., Kamata, M., *Jap. J. Microb.*, 1957, v1, 43.

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Metabolism of Thiamine-S^{35} in the Rabbit.* (24091)

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McCarthy, Cerecedo, and Brown(1) showed that rats which had received an intramuscular injection of thiamine-S^{35} excreted about 64% of the radiosulfur in urine, and between 1 and 2% in feces, within 10 days after administration. Isolation of thiamine-S^{35} from urine by adsorption on synthetic zeolite Decalso showed that approximately 60% radiosulfur of the neutral sulfur fraction was contained in unchanged thiamine-S^{35}, and the remaining 40% was present in neutral sulfur compounds not adsorbed by Decalso. The present study was based on use of doses of radiovitamin that were in the physiological range, *i.e.*, from 50 to 200 γ administered by oral and parenteral routes, to ascertain whether different routes of administration would lead to gross differences in the metabolic pattern.

Materials and methods. Metabolic studies were carried out using Chinchilla or Dutch breed adult rabbits. These were housed in wire-bottom metabolism cages allowing complete separation of urine and feces, and were given Purina Rabbit Chow and water *ad lib.* with supplement of lettuce and carrots once or twice weekly except immediately prior to and during experimental periods. Each individual experiment was carried out with 2 rabbits, and in so far as possible, the same pair of rabbits was used in successive experiments so that, in effect, each rabbit was its own control. Urine samples were collected in 10 ml of glacial acetic acid as a preservative which brought the pH from range 9-10 to 4-5, in which thiamine-S^{35} and its metabolites would be more stable. Urine samples were filtered and diluted with distilled water. Feces were homogenized with 150 ml of water containing about 10 ml of concentrated hydrochloric acid. The homogenate stood several hours, was stirred occasionally, and finally centrifuged to remove the bulk of solid matter. The supernatant was filtered and diluted with distilled water. Radiosulfur of urine was determined by analysis of 3 sulfur fractions: inorganic sulfate, ethereal sulfate, and neutral sulfur. Fecal sulfur was partitioned into inorganic sulfate and neutral sulfur fractions. The method of Folin was utilized for isolation of inorganic sulfate, and total sulfate (inorganic plus ethereal) fractions, while the neutral sulfur fraction was obtained by subtracting total sulfate from total sulfur, as determined by the Denis modification of the method of Benedict (2a). All sulfate fractions were precipitated as barium sulfate, and filtered onto porous glass frits mounted in stainless steel holders. Precipitates were washed successively with water, methanol, and acetone, and dried at 110°C for one hour. Samples were counted in a Nuclear windowless or thin-window flow-gas Geiger-Mueller counter and scaler. All count rates were corrected for background, counter efficiency, decay of sulfur-35, and self-absorption. A sample of barium sulfate-S^{35} was prepared from an amount of thiamine-S^{35} identical to that administered to rabbits. This standard sample was counted each day that experimental samples were counted, and comparison of count rates of samples with this standard gave the percent recovery of the radiosulfur. In addition to sulfur analyses, urea excreted in urine was determined each day by the colorimetric method of Rosenthal, using diacetyl monoxime in arsenic acid(3). Under normal conditions of nutrition, daily excretion of urea parallels that of inorganic sulfate in urine(2b). Therefore urea : inorganic sulfate ratios were calculated daily to ascertain that no marked alterations occurred in nitrogen equilibrium. Preliminary experiments indicated that unchanged thiamine-S^{35} and its thiazole-S^{35} moiety were main metabolites of radiovitamin in rabbit urine, and procedures were devised to isolate these substances. A modification of the method of Herr(4) was used for isolation of thiamine-

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TABLE I. Percent Recovery of S^{35} in Urine and Feces of Rabbits after Administration of 100 γ of Thiamine-S^{35} by Stomach Tube.

24-hr excretion period	Fraction	Urine		Feces	
		Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2
1st	Inorganic sulfate	2.60	1.29	2.10	.47
	Ethereal "	.92	1.37		
	Neutral sulfur	37.85	43.22	7.39	5.92
	Total	41.37	45.88	9.49	6.39
2nd	Inorganic sulfate	.74	.42	.57	.90
	Ethereal "	.42	.00		
	Neutral sulfur	3.26	2.28	2.37	1.35
	Total	4.42	2.70	2.94	2.25
3rd	Inorganic sulfate	.59	.42		
	Ethereal "	.11	.00		
	Neutral sulfur	2.99	.96		
	Total	3.69	1.38	1.48	.90
4th	Inorganic sulfate	.11	.21		
	Neutral sulfur	.88	.26		
	Total	.99	.47	.73	.55
5th	Total	.67	.21		
	Total recovery	51.12	50.64	14.64	10.09

Total recovery, urine and feces: Rabbit 1, 65.76%; Rabbit 2, 60.73%.

S^{35}, in which it was removed from urine by adsorption on column of strong cation exchange resin Dowex-50-X8 with subsequent elution by concentrated hydrochloric acid. Recovery experiments indicated that between 80 and 90% of thiamine-S^{35} could be removed from urine by this procedure. The hydrochloric acid eluate was evaporated to dryness over soda lime and phosphorus pentoxide in vacuum desiccator; the residue was taken up in about 10 ml of anhydrous methanol, the resulting solution filtered, and the filtrate evaporated to dryness. The final residue was dissolved in 5 to 8 ml of anhydrous methanol, and an aliquot of this solution was used for determination of the radiosulfur content. Thiazole-S^{35} was extracted from urine with ether. Prior to extraction, about 25 mg of non-radioactive thiazole were added to urine to act as carrier, the urine adjusted to pH 10 by addition of sodium hydroxide, then extracted with ether in a continuous extractor for 12 to 16 hours. The ethereal extract was dried over anhydrous magnesium sulfate, and ether distilled off. The thiazole-S^{35} residue was dissolved in 5 to 8 ml of anhydrous ethanol, and an aliquot used for determination of radiosulfur content. The presence of these metabolites in the extracts was confirmed by paper chromatography on strips of Whatman #1 paper (1.5 x 22 inches) in the solvent system n-butanol-acetic acid-water (4 : 1 : 1). Ascending chromatography was used. The chromatograms were developed 8 to 10 hours in the solvent system, then dried at room temperature. Spots were identified by inspecting the chromatograms in ultraviolet light and by scanning the strips in a windowless flow-gas Geiger-Mueller counter designed for this purpose (Anderson Scannergram). Thiamine-S^{35} bromide hydrobromide was prepared from thiourea-S^{35} (5).†

Results. Oral administration. In experiments in which thiamine-S^{35} was administered to rabbits by oral route, 3 dose levels in the physiological range were used: 50, 100, and 200 γ. The thiamine-S^{35} (about 25,000 counts/min/100 γ) was dissolved in 1 ml of water and administered to rabbits by stomach tube. The excretion pattern was the same for all 3 levels; however, total recovery of radiosulfur in urine varied from 50 to 90% in 4 to six 24-hour periods following administration of radiovitamin. In all cases, more than 50%

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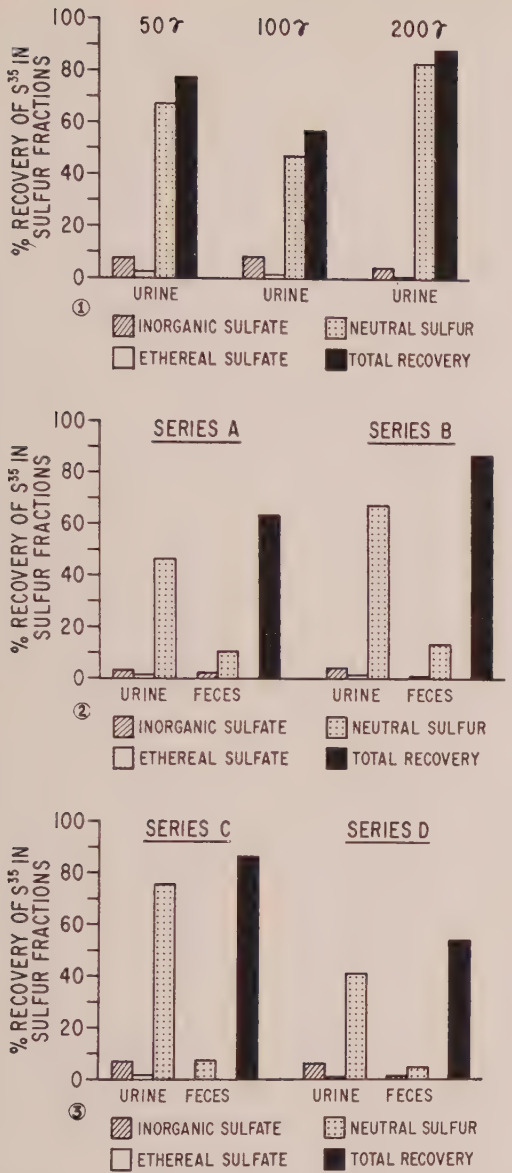

FIG. 1. Oral administration of thiamine-S^{35} to rabbits. Recovery of radiosulfur in urinary sulfur fractions (five 24-hr periods) after administration of 50, 100 and 200 γ of thiamine-S^{35} by stomach tube.

FIG. 2. Oral administration of thiamine-S^{35} to rabbits. Recovery of radiosulfur in urine and feces: Series A, 100 γ of thiamine-S^{35} by stomach tube; Series B, 100 γ of thiamine-S^{35} by stomach tube, followed 6 hr later by intramusc. inj. of 2 mg of non-radioactive thiamine. (Urine: five 24-hr periods; feces: four 24-hr periods.)

FIG. 3. Parenteral administration of thiamine-S^{35} to rabbits. Recovery of radiosulfur in urine and feces: Series C, 100 γ of thiamine-S^{35} by intramusc. inj.; Series D, 100 γ of thiamine-S^{35} by intrav. inj. (Urine: six 24-hr periods; feces: four 24-hr periods.)

of the recovered radiosulfur was in the neutral sulfur fraction of the urine, and the greater portion of this was recovered in urine excreted in the first 24-hour period. The second 24-hour period usually contained half, or less than half, of the amount of radiosulfur recovered in the first period, and again this was contained primarily in the neutral sulfur fraction. Radiosulfur was found in continuously diminishing amounts in succeeding excretion periods. Inorganic sulfate and ethereal sulfate fractions of urine contained very small amounts of radioactivity. Feces contained 8 to 14% of administered radiosulfur, present primarily in neutral sulfur compounds. Table I contains data from typical experiments in which 100 γ of thiamine-S^{35} were administered to rabbits, and radiosulfur content of urinary and fecal sulfur fractions determined. Average recoveries of radiosulfur, obtained in urine fractions when the 3 dose levels were administered, are presented in Fig. 1.

In further experiments involving oral administration of thiamine-S^{35}, the oral dose of 100 γ of radiovitamin was followed 6 hours later by intramuscular injection of 2 mg of non-radioactive thiamine. This was done to determine if injection of the 20-fold excess of non-radioactive thiamine would cause increased excretion of radiosulfur from labeled vitamin. This increased excretion of radiosulfur, due to flushing out effect of excess non-radioactive thiamine injected was, in fact, realized, and an additional amount (average 17%) of administered radiosulfur was recovered. Practically all of this additional radiosulfur excreted was found in the neutral sulfur fraction of urine in the first 24-hour excretion period. Urinary and fecal recoveries of radiosulfur are shown in Fig. 2. For comparison, data are also presented from experiments in which 100 γ of thiamine-S^{35} were administered orally to rabbits without follow-up injection of non-radioactive thiamine, and urinary and fecal recoveries of radiosulfur determined for the same time. In contrast to the marked difference in excretion of radiosulfur in the neutral sulfur fraction of urine in these 2 series of experiments, inorganic sulfate and ethereal sulfate fractions of urine,

and fecal sulfur fractions show no significant differences in radiosulfur recovery.

In oral administration shown in Fig. 2, the main metabolites of thiamine-S^{35} were isolated from urine collected in the first 24-hour excretion period. It was found that thiamine-S^{35} accounted for approximately 60 to 65%, and thiazole-S^{35} accounted for approximately 34% of neutral sulfur. These results are similar to those obtained by McCarthy *et al.*(1), and by Iacono and Johnson(6), who found that when rats were given intraperitoneal injection of thiazole-2-C^{14}-thiamine, unchanged radiothiamine accounted for about 60% of radiocarbon of urine.

Parenteral administration. The radiovitamin was administered to rabbits by intramuscular injection, and by intravenous injection. In both series, thiamine-S^{35} was dissolved in normal saline and pH of solution adjusted to 7.0. Injections of 100 γ were given in the musculature of hind leg, and in marginal ear vein, respectively. The results are presented in Fig. 3.

When radiovitamin was administered by intramuscular injection, average total recovery of radiosulfur in urine and feces was 86% for six 24-hour periods. Radiosulfur recovered in neutral sulfur accounted for 75% of administered dose. Average total recovery of radiosulfur, for the same time, when radiovitamin was administered by intravenous injection, was 55%, of which about 41% was in the neutral sulfur fraction of urine. For both parenteral routes, as with oral administration, inorganic and ethereal sulfate fractions of urine, and fecal sulfur fractions are minor excretory pathways for radiovitamin and its metabolites.

The results of our intramuscular injection experiments are similar to those obtained by other workers who studied the fate of intramuscularly injected thiamine-S^{35} in other species. McCarthy and coworkers(1) obtained essentially the same results after intramuscular injection of 50 γ of thiamine-S^{35} to rats. Borsook and his associates(7), though working with pharmacological doses of radiovitamin, obtained similar results with a human subject. Six days after the last of 4 daily injections of thiamine-S^{35} (16 mg), 61% of radiosulfur had been recovered in urine, and 11% in feces.

Studies on fate of intravenousliy injected radioactive thiamine have not been reported. As data in Fig. 3 indicate, recovery of radiosulfur is considerably lower than that obtained by either of the other routes used. A possible explanation is that, since radiovitamin is introduced directly into the bloodstream, it may be removed from the blood and stored by liver and other organs to a larger extent than when administered orally or intramuscularly, and is therefore retained and excreted over longer periods of time.

Summary. The fate of thiamine, labeled with sulfur-35 in the thiazole moiety, has been studied in rabbits after oral and parenteral administration of doses in the physiological range. Analyses of the urinary and fecal sulfur fractions for 4 to six 24-hour periods following administration of radiovitamin, gave average total recovery of radiosulfur of 77% after administration by stomach tube, 86% after intramuscular injection, and 54% after intravenous injection. When oral dose of radiovitamin was followed 6 hours later by intramuscular injection of a 20-fold larger dose of non-radioactive thiamine, an additional 17% of radiosulfur was recovered in urine. In all cases, the neutral sulfur fraction of urine contained more than 50% of the recovered radiosulfur, and the greater portion of this was excreted in the first 24-hour period following administration of radiovitamin. Isolation of main metabolites following oral administration indicates that unchanged thiamine-S^{35} and thiazole S^{35} moiety together account for approximately 95% of radiosulfur of the neutral sulfur fraction in the first 24-hour period, and are excreted in a ratio of 2 : 1, respectively.

1. McCarthy, P. T., Cerecedo, L. R., Brown, E. V., *J Biol. Chem.*, 1954, v209, 611.

2. Hawk, P. B., Oser, B. L., Summerson, W. H., *Practical Physiological Chemistry,* McGraw-Hill Book Co., 13th ed., 1954, (a) p946; (b) p948.

3. Rosenthal, H., *Anal. Chem.*, 1955, v27, 1980.

4. Herr, D. S., *Ind. Eng. Chem.*, 1945, v37, 631.

5. Verrett, M. J., Cerecedo, L. R., *J. Org. Chem.*, 1957, v22, 1695.

6. Iacono, J. M., Johnson, B. C., *J. Am. Chem. Soc.*, 1957, v79, 6321.

7. Borsook, H., Buchman, E. R., Hatcher, J. B., Yost, D. M., McMillan, E., *Proc. Nat. Acad. Sci.*, 1940, v26, 412.

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Immunological Characteristics of N. Y. Strains of Influenza A Virus From the 1957 Pandemic. (24092)

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A close antigenic relationship among influenza A virus strains isolated in various parts of the world during the 1957 pandemic has been demonstrated(1), and the large differences between these strains and earlier strains have been emphasized(2,3). However, evidence that there is slight antigenic relationship between strains prevalent in 1957 and older strains has been steadily accumulating. Mulder(4) first reported the presence of antibody to the new strains, in persons over 70 years of age, and this was confirmed by Davenport(5). Patients with influenza in various locations have occasionally shown rises in antibody titer to older strains(1,2). Monovalent Asian vaccine has produced antibody response to older prototype strains(6), and polyvalent vaccine not containing an Asian strain has produced a rise in titer to Asian strains in patients over 70(7). One of the problems in detecting infection and in evaluating antibody response with Asian strains has been the low hemagglutination-inhibiting titers obtained with Japan/305/57 strain. The 1957 influenza pandemic reached its peak in N. Y. City in October. From September to November 6 strains of influenza A virus were isolated from patients at the Hospital of the Rockefeller Institute. In the present communication the immunological characteristics of these strains are described. Evidence is presented that the new strains contain an antigenic component that has been present in previous influenza A virus strains. The reactivity of viruses from the 1957 pandemic with antibody and non-specific inhibitors of hemagglutination is described. One of the new isolates is a highly sensitive indicator of the presence of hemagglutination-inhibiting and neutralizing antibodies in human serum. This property has made the Rockefeller Institute/5/57 strain especially useful in diagnosing infection with recently prevalent influenza viruses, in demonstrating antibody against them in human sera obtained before the 1957 epidemic, and in detecting an immunological response to monovalent Asian influenza vaccine.

Materials and methods. Viruses. The 1957 New York isolates were recovered in embryonated eggs by combined amniotic and allantoic inoculation of throat washings. The new strains were designated in order of isolation A/Rockefeller Institute/1/57 to A/Rockefeller Institute/6/57. The viruses were used after 2 to 4 passages in the allantoic cavity. The Far East isolates Japan/305/57 and Formosa/313/57(2) were kindly provided by Miss M. L. Miesse of the Walter Reed Army Institute of Research. The strain A/New York/1/53 was isolated at the Rockefeller Institute. The strains Swine/15, PR8, FM1, and Cuppett are well known prototype strains. *Rabbit sera.* Rabbit antisera against the 1957 isolates and Swine/15 were prepared by intravenous injection of 10 ml of virus infected allantoic fluid, followed, in 2 weeks, by intraperitoneal injection of 10 ml of the same material. The rabbits were bled 2 weeks after the second injection. Antisera against PR8, FM1, Cuppett and New York/1/53 had been previously prepared in this laboratory, and stored at 4°C. In each case a pool of sera from 2 rabbits was employed. *Patients' sera.* Acute sera and convalescent sera collected 14 to 21 days from onset of illness were obtained

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from a series of patients with the clinical picture of influenza. An additional later convalescent serum was obtained from some patients. After standing overnight at 4°C, serum was separated from coagulated blood and stored at –20°C. *Red blood cells.* Suspensions of chicken red blood cells, standardized photometrically, were used in hemagglutination and hemagglutination-inhibition titrations at a final concentration of 0.18% by packed cell volume. *Hemagglutination titrations.* Glass test tubes with an internal diameter of 10 mm and hemispherical bottoms were used. Serial 2-fold dilutions of infected allantoic fluids were made in phosphate buffered saline. To 0.5 ml of each dilution was added 0.5 ml of 0.36% chicken rbc suspension. Reactions were read after 70 minutes, and the end point was considered to be in the highest dilution showing marked but not complete agglutination (2+) of rbc. *Hemagglutination-inhibition titrations.* Human sera were inactivated at 56°C for 30 minutes. Rabbit sera, diluted with equal volumes of phosphate buffered saline, were inactivated at 65°C for 30 minutes and absorbed at 4°C with 20% chicken rbc. Both human and rabbit sera were treated(8) with *V. cholerae* filtrate to remove non-specific inhibitors of hemagglutination. Serial 2-fold dilutions of inactivated serum were made in saline. To 0.3 ml of each dilution was added 0.3 ml of infected allantoic fluid diluted so as to give a final concentration of 4 hemagglutinating units of virus in each tube. To each mixture was added 0.6 ml of 0.36% suspension of chicken rbc. Readings were made after 70 minutes at room temperature and the end point was taken as the highest dilution of serum which completely inhibited rbc agglutination. Titers are expressed as the reciprocal of the final dilution. In some titrations of human serum a lowest final dilution of 1:4 was obtained by mixing 0.6 ml of undiluted serum with 0.3 ml of diluted infected allantoic fluid, and adding 0.3 ml of 0.72% chicken rbc. *Neutralization titrations.* Human sera were inactivated at 56°C for 30 minutes prior to testing. *In ovo* neutralization titrations were carried out in 10 or 11 day old chick embryos as previously described(9), except that the inoculum of 1000 EID_{50} per egg was contained in a volume of 0.1 ml of the serum-virus mixture. *Computations.* To determine the extent of the immunological relationship among strains, serum titer ratios $\left(r = \frac{\text{heterologous titer}}{\text{homologous titer}} \right)$ were used to compute R values according to the expression, $R = \sqrt{r_1 \times r_2}$(10). The R values, multiplied by 100(9), provide estimates of immunological relationship between any 2 strains in terms of per cent. *Antibody absorption.* Virus-erythrocyte complexes were prepared according to the procedure described by Jensen and Francis(11). In absorption experiments mixtures of virus-erythrocyte complexes and *V. cholerae* filtrate treated sera were rocked for 30 minutes at a rate of 8 oscillations per minute at 5°C. The rbc were then sedimented at 1000 rpm for 10 minutes. The sera were collected and antibody content measured by hemagglutination inhibition. Experiments showed that absorption was maximal in 30 minutes.

Results. Cross hemagglutination-inhibition titrations with 1957 and prototype strains of influenza A virus and rabbit antisera. As shown in Table I, all of the 1957 isolates were antigenically closely related. Furthermore, there was evidence of slight cross-relationship between some of the 1957 strains and some of the older prototype strains. In numerous instances the relationship was evident in only one part of the cross test, but in others it was seen in both parts. The extent of immunological relationship, expressed in per cent, is shown in Table II. The close immunological relationship among all of the 1957 strains is again readily apparent. The slight but definite relationship of a 1957 isolate to an older strain is illustrated by RI/5/57† and FM1, in which case R x 100 = 2.2%. For comparison, the same degree of relatedness was found between PR8 and FM1. In all hemagglutination-inhibition titrations with immune rabbit sera, corresponding pre-immunization sera were used as controls for the presence and level of non-specific inhibitors. In a few instances non-specific inhibitory activity was

† RI = Rockefeller Inst.

TABLE I. Cross Hemagglutination-Inhibition Reactions with 1957 Isolates and Prototype Strains of Influenza A Virus.

	Virus											
	Far East		New York						Prototype strain			
Rabbit antiserum	Japan /305	Formosa /313	RI/1/57*	RI/2/57	RI/3/57	RI/4/57	RI/5/57	RI/6/57	Swine/15 (1931)	PR8 (1934)	FM1 (1947)	Cuppett (1950)	NY/1 (1953)
	Serum hemagglutination-inhibition titer											
Japan/305/57	*2048*	512	8192	4096	4096	2048	2048	4096	<16	16	16	16	<16
Formosa/313/57	1024	*1024*	4096	2048	"	"	"	2048	"	"	32	<16	16
RI/1/57	"	256	*4096*	"	"	"	"	"	"	"	16	"	<16
2	"	"	"	*2048*	"	"	"	"	"	"	"	"	"
3	"	"	8192	4096	*4096*	"	"	"	32	32	32	"	16
4	"	"	"	"	8192	*2048*	"	4096	<16	"	"	16	32
5	256	64	4096	1024	4096	1024	*2048*	1024	32	"	64	"	"
6	512	128	"	2048	"	"	"	*2048*	<16	‡	16	"	16
Swine/15 (1931)	<16	<16	32	<16	<16	‡	32	<16	*2048*	128	32	32	32
PR8 (1934)	"	"	"	"	"	32	"	"	<16	*8192*	"	<16	"
FM1 (1947)	"	"	16	"	"	"	16	"	"	128	*1024*	512	256
Cuppett (1950)	"	"	<16	"	"	16	<16	"	"	16	4096	*4096*	512
NY/1 (1953)	"	"	"	"	"	<16	"	"	"	256	512	64	*8192*
	Titer ratio†											
Japan/305	*1*†	1/4	4	2	2	1	1	2	<1/128	1/128	1/128	1/128	<1/128
Formosa/313	1	*1*	4	2	4	2	2	2	<1/64	1/64	1/32	<1/64	1/64
RI/1/57	1/4	1/16	*1*	1/2	1	1/2	1/2	1/2	<1/256	1/256	1/256	<1/256	<1/256
2	1/2	1/8	2	*1*	2	1	1	1	<1/128	1/128	1/128	<1/128	<1/128
3	1/4	1/16	2	1	*1*	1/2	1/2	1/2	1/128	1/128	1/128	<1/256	1/256
4	1/2	1/8	4	2	4	*1*	1	2	<1/128	1/64	1/64	1/128	1/64
5	1/8	1/32	2	1/2	2	1/2	*1*	1/2	1/64	1/64	1/32	1/128	1/64
6	1/4	1/16	2	1	2	1/2	1	*1*	<1/128	‡	1/128	1/128	1/128
Swine/15 (1931)	<1/128	<1/128	1/64	<1/128	1/128	‡	1/64	<1/128	*1*	1/16	1/64	1/64	1/64
PR8 (1934)	<1/512	<1/512	1/256	<1/512	<1/512	1/256	1/256	<1/512	<1/512	*1*	1/256	<1/512	1/256
FM1 (1947)	<1/64	<1/64	1/64	<1/64	<1/64	1/32	1/64	<1/64	<1/64	1/8	*1*	1/2	1/4
Cuppett (1950)	<1/256	<1/256	<1/256	<1/256	<1/256	1/256	<1/256	<1/256	<1/256	1/256	1	*1*	1/8
NY/1 (1953)	<1/512	<1/512	<1/512	<1/512	<1/512	<1/512	<1/256	<1/512	<1/512	1/32	1/16	1/128	*1*

* RI = Rockefeller Inst. † Titer ratio = heterologous titer/homologous titer. ‡ Non-specific inhibitor not completely eliminated.

TABLE II. Comparison of Extent of Immunological Relationships among 1957 Far East and New York Isolates and Prototype Strains of Influenza A Virus as Shown by the Hemagglutination-Inhibition Procedure.

Rabbit antiserum	Far East		New York						Prototype strain				
	Japan /305	Formosa /313	RI/1/57	RI/2/57	RI/3/57	RI/4/57	RI/5/57	RI/6/57	Swine/15 (1931)	PR8 (1934)	FM1 (1947)	Cuppett (1950)	NY/1 (1953)
Japan/305/57	*100**												
Formosa/313/57	50	*100*											
RI/1/57	100	50	*100*										
2	"	"	"	*100*									
3	71	"	141	141	*100*								
4	"	"	"	"	141	*100*							
5	35	25	100	71	100	71	*100*						
6	71	35	"	100	"	100	71	*100*					
Swine/15 (1931)	<.8	<1.1	<.8	<.8	.8	†	1.6	<.8	*100*				
PR8 (1934)	<.4	<.6	<.4	<.4	<.4	.8	.8	†	<1.1	*100*			
FM1 (1947)	<1.1	<2.2	<.8	<1.1	<1.1	2.2	2.2	<1.1	<1.6	2.2	*100*		
Cuppett (1950)	<.6	<.8	<.4	<.6	<.4	.6	.6	<.6	<.8	<.3	71	*100*	
NY1 (1953)	<.4	<.6	<.3	<.4	<.3	<.6	<.8	<.4	<.6	1.1	17	3.1	*100*

* Expressed as per cent of immunological relationship; $R \times 100 = 100\sqrt{r_1 \times r_2}$. † Could not be calculated because of non-specific inhibition.

not reduced below a titer of 1:16 by the procedure used. However, residual non-specific inhibitory activity interfered with determination of antibody levels only in 2 instances, as indicated in Table I. In these cases the hemagglutination-inhibiting activity of pre- and post-immunization sera was equally low. In certain other cases, the hemagglutination-inhibiting activity of post-immunization sera was much higher (8 times or greater) than that of the pre-immunization sera, therefore it was concluded that high inhibitory activity in post-immunization sera represented antibody. These cases involved the RI/4/57 strain and rabbit sera of recent origin. Pre-immunization controls of antisera against the prototype strains lacked inhibitory activity against RI/4/57. These sera had been prepared several years earlier and had been stored at 4°C.

Hemagglutination-inhibiting antibody levels in sera from cases of influenza. Table III summarizes the results obtained with paired sera from 13 patients seen in the fall of 1957. From 6 of these patients virus was isolated. It can be seen that no patient showed antibodies against Japan/305/57 in acute phase serum and that only 5 showed a rise of low degree in convalescent serum. In marked contrast, 4 patients showed antibodies against RI/5/57 in acute phase serum and 12 showed a moderate to marked rise in convalescent serum. The titers in convalescent serum against the other 1957 isolates were higher than those against Japan/305/57, but considerably lower than those against RI/5/57. The latter was true, with one exception, even when the serum titer against the homologous isolate was compared to that against RI/5/57. Two other patients, ET and JV, with typical responses to the 1957 strains showed a rise to Swine/15 and one, MB, showed a rise to FM1. One patient, IB, age 62, was unique in showing no rise to any 1957 strain, but a considerable rise against Swine/15 and PR8, and an even greater rise against FM1.

A third serum specimen was obtained from some patients approximately 2 months from the onset of illness. On the average a 4-fold decrease from the second specimen antibody level was observed in these sera.

TABLE III. Hemagglutination-Inhibiting Antibody Rise in Patients with Influenza, Fall 1957, New York.

			Hemagglutination-inhibition titer								
			Influenza A virus strain								
			1957						1931	1934	1947
Patient	Age	Serum	Japan /305	RI/1	RI/2	RI/3	RI/5	RI/6	Swine/15	PR8	FM1
RG	19	A	<8	<16	<8	<16	32	<16	<16	1024	2048
(RI/1/57)*		C	"	*512*	64	128	2048	512	"	"	"
ET	42	A	"	<16	<8	<16	<8	<16	32	64	16
(RI/2/57)*		C	"	256	*8*	64	1024	128	256	"	"
EC	23	A	"	<16	<8	<16	<8	<16	<8	512	512
(RI/3/57)*		C	"	256	32	*128*	1024	256	"	"	"
Tuc	24	A	"		<8		<8			4096	"
(RI/4/57)*		C	16		64		2048			"	"
PC	28	A	<8	<16	<8	<16	<8	<16	<8	1024	256
(RI/5/57)*		C	"	"	"	"	*32*	"	"	"	"
LW	21	A	"	"	"	"	32	"	<16		
(RI/6/57)*		C	8	256	64	128	256	*512*	"		
VG	21	A	<8		<8		<8			512	2048
		C	"		32		256			"	"
MV	20	A	"	<16	<8	<16	<8	<16	<8	"	512
		C	16	512	128	256	8192	256	"	"	"
JV	25	A	<8	<16	<8	<16	<8	<16	<16	1024	1024
		C	32	256	64	64	1024	128	32	"	"
IB	62	A	<8		<8	<16	64	<16	<8	128	32
		C	"		"	"	"	"	128	1024	1024
MB	58	A	"		"		<8		512	"	128
		C	16		32		1024		"	"	512
SV	27	A	<8	<16	<8	<16	<8	<16	1024	8192	4096
		C	"	8192	1024	4096	32,768	16,384	"	"	"
SO	32	A	"	<16	<8	<16	16	<16	128	2048	64
		C	"	32	"	16	256	16	"	"	"

* Virus isolated. A = acute; C = convalescent.

Results of titrations with RI/4/57 and patients' sera are not shown because attempts to eliminate non-specific inhibitors of hemagglutination for this strain were unsuccessful. With all the other strains *V. cholerae* filtrate treatment of serum was effective.

TABLE IV. Neutralizing Antibody Rise in Patients with Influenza, Fall 1957, New York.

		In ovo neutralization titer	
		1957 influenza A virus strain	
Patient's serum		RI/2	RI/5
ET	Acute	<8	15
(RI/2/57)*	Convalescent	"	53
EC	Acute	"	<8
(RI/3/57)*	Convalescent	"	64
PC	Acute	"	<8
(RI/5/57)*	Convalescent	"	29
JV	Acute	"	12
	Convalescent	20	64
SO	Acute	<8	<8
	Convalescent	"	28

* Virus isolated.

Neutralizing antibody levels in sera from cases of influenza. *In ovo* neutralization tests (Table IV) demonstrated the presence of neutralizing antibodies against RI/5/57 in acute phase serum from 2 of 5 patients, and failed to reveal any against RI/2/57. A rise in antibody titer was found in all of the 5 patients with RI/5/57 as the test virus, but only in one of the 5 with RI/2/57. These findings confirmed the results of hemagglutination-inhibition titrations.

Hemagglutination-inhibiting antibody levels in sera obtained before the 1957 epidemic. Because of the finding of antibodies to RI/5/57 in the acute phase sera of patients, an

TABLE V. Presence of Hemagglutination-Inhibiting Antibodies against 1957 Isolates and Prototype Strains of Influenza A Virus in 24 Human Sera, 1940-1957. Japan 305 (Far East) and RI/2/57 (N. Y.) are all <4.

Serum		Hemagglutination-inhibition titer — Influenza A virus strain			
		New York	Prototype strains		
Computed present age	Year bled	RI/5/57	Swine/15 (1931)	PR8 (1934)	FM1 (1947)
72	1940	64	<8	64	8
37	"	8	<8	<16	<8
35	1942	128	<8	128	16
*	"	8	8	32	<8
45	"	4	<8	<8	<8
*	1943	8	<8	8	<8
37	"	16	128	512	8
45	"	4	16	16	<8
48	1944	4	16	32	16
29	1945	<4	<8	64	16
35	"	4	8	<8	<8
39	1947	64	16	32	32
53	1948	4	32	512	16
39	"	<4	<8	16	<8
54	"	4	<8	64	16
26	1951	<4	8	2048	512
46	1952	<4	8	128	16
48	1953	32			
71	"	16	32	512	512
40	1954	<4	8	128	16
57	1955	<4	8	256	8
35	1956	16	64	32	32
17	1957	128	<8	1024	1024
62	"	<4	<8	64	64

* Not known.

investigation of antibody levels of sera which had been obtained from 1940 until May 1957 was carried out. These individual sera were obtained from 24 patients with various diseases and had been stored at 4°C. Table V shows that in 24 sera no antibodies were demonstrated to Japan/305/57 or to RI/2/57. In contrast, 17 sera contained antibodies to RI/5/57, though in some cases the concentration was very low. The computed present ages of these patients varied from 17 to 72. There was no apparent correlation between antibody titers to RI/5/57 and those to the older prototype strains.

Antibody response to monovalent Asian influenza vaccine. The finding of high antibody levels to RI/5/57 as compared to other strains in sera of patients with influenza led to an examination, with RI/5/57, of antibody response of 3 normal adults who had received a monovalent vaccine containing 200 CCA units per ml of Japan/305/57 strain. Each had received 2 subcutaneous injections of 1 ml 3 weeks apart, and had been bled 2 weeks after the second injection. Table VI shows that in the vaccinated persons a greater response was demonstrated with RI/5/57 than with Japan/305/57 or RI/2/57. As described above, a similar observation was made in cases of influenza.

Sensitivity to non-specific inhibitors of hemagglutination. The insensitivity of Japan/305/57 to non-specific inhibitors has been described(2,3). The relative sensitivity of the 1957 New York strains to non-specific inhibitors of hemagglutination present in human and rabbit sera treated by heating alone was determined in experiments summarized in Table VII. The human serum used was obtained 2 months prior to the onset of the 1957 epidemic in New York, and after treatment with *V. cholerae* filtrate no antibodies were demonstrated in this serum to any of the 1957 strains. It was found that 3 of the 6 New York strains were insensitive, one was slightly sensitive, and 2, RI/4/57 and RI/5/57, were highly sensitive.

Antibody absorption. The low titers in pa-

TABLE VI. Hemagglutination-Inhibiting Antibody Rise in Persons Vaccinated with Formalin-Treated Influenza A Virus, Japan/305/57 Strain.

Vaccinee's serum		Hemagglutination-inhibition titer — Influenza A virus strain					
		Japan/305	1957 RI/2	1957 RI/5	1931 Swine/15	1934 PR8	1947 FM1
VQ	Before	<8	<8	<8	8	64	64
	After	8	8	32	"	"	"
BL	Before	<8	<8	"	<8	1024	128
	After	"	16	256	"	"	"
HS	Before	"	<8	<8	32	128	<8
	After	"	8	64	"	"	8

TABLE VII. Sensitivity of 1957 Influenza A Strains to Non-Specific Inhibitors of Hemagglutination in Human and Rabbit Serum.

Virus	Hemagglutination-inhibition titer: Human serum* lacking antibodies against 1957 strains	Hemagglutination-inhibition titer: Normal rabbit serum†
Japan/305/57	<8	<8
RI/2/57	"	"
3	"	"
6	"	"
1	16	32
5	512	2048
4	1024	8192

* Heated at 56°C for 30 min.
† " " 65°C " " .

tients' sera against homologous isolates, as compared to the high titers against RI/5/57, suggested the possibility that these strains had stimulated production of antibody with which they could not react efficiently. Fiset and Depoux(12) showed that some strains of influenza A virus in Q phase were capable of stimulating production in ferrets of antibodies which they were unable to absorb. To test this possibility with 1957 New York strains, antibody absorption experiments were done using as the absorbing antigen the complex formed by periodate treated virus and formalin treated human erythrocytes(11). The 2-month convalescent sera of the patients from which RI/2/57 and RI/5/57 were isolated were cross absorbed with these 2 strains. It was found that RI/2/57 and RI/5/57 could each absorb completely the antibodies against both strains from either serum if a sufficient quantity of antigen was used.

Discussion. The results of the cross hemagglutination-inhibition reactions indicate that, like other later strains isolated during the 1957 pandemic(1), the New York isolates are closely related among themselves and to the Far East strains isolated earlier in the year. The cross reactions also provide evidence for an antigenic component common to the 1957 strains and older prototype strains. Further evidence on this point is provided by the finding of antibody to RI/5/57 in acute phase sera of some influenza patients, in rises in titer of a few patients to the older strains, and in the finding of antibody levels to RI/5/57 in the sera collected from 1940 to 1957.

To overcome the difficulties which have been encountered in detecting infection and in evaluating response to vaccination with Asian strains because of the low hemagglutination-inhibiting titers obtained with Japan/305/57 strain, the use of an egg-ferret-mouse-egg line of Japan/305/57 has been recommended(1). This line gives higher titers, but is sensitive to non-specific inhibitors and requires periodate treatment of sera. Others(6) have found that titers obtained with this animal line and periodate or *V. cholerae* filtrate treated sera are not significantly higher than those obtained with an egg line of Japan/305/57 and untreated sera. In the present study a strain, RI/5/57, was found, which gives high hemagglutination-inhibiting and neutralizing antibody titers with human sera. This strain has the advantage of having been passed in eggs only. As is well known, passage in the mouse lung may lead to unpredictable alterations in the antigenic characteristics of influenza virus strains(13,14,15), whereas on egg passage the antigenic properties have been found to be stable(10,15,16). It should be emphasized that treatment of human serum with *V. cholerae* filtrate eliminates non-specific hemagglutination-inhibiting activity against RI/5/57. The usefulness of RI/5/57 in diagnosing infection with the recently prevalent influenza viruses, in demonstrating antibody in human sera collected prior to the 1957 epidemic, and in detecting a response to monovalent Asian influenza vaccine has been documented here. The results obtained indicate that unless agreement is reached concerning test antigens to be used in various studies, results will not be comparable and may be misleading.

The mechanism of difference in serological reactivity of antigenically similar influenza strains is not clear. Differences in steric arrangement of antigens has been suggested(17, 18). The possibility of a masked antigen capable of stimulating antibody production but unable to combine with it in antibody absorption experiments has been described(12). This was shown not to be the case with the RI/2/57 strain by antibody absorption experiments which demonstrated that RI/2/57 virus has the capacity to absorb all of the

antibody present to itself and to RI/5/57.

Summary. Six strains of influenza A virus were isolated in New York during the 1957 pandemic. Close antigenic relationship was found among these strains and 2 Far East isolates, and slight antigenic relationship to older prototype strains was also demonstrated. One of the local isolates, RI/5/57, was found to be a very sensitive indicator of the presence of hemagglutination-inhibiting and neutralizing antibodies against the 1957 strains of influenza A virus in human serum. Antibodies to the RI/5/57 strain were demonstrated in patients of various ages obtained from 1940 to 1957. The sensitivity of the 6 New York strains to non-specific inhibitors of hemagglutination varied from apparent insensitivity in 3 strains to extreme sensitivity in 2.

1. Jensen, K. E., Hogan, R. B., *Pub. Health Rep.,* 1958, v73, 140.

2. Meyer, H. M., Jr., Hilleman, M. R., Miesse, M. L., Crawford, I. P., Bankhead, A. S., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 609.

3. Jensen, K. E., *J.A.M.A.,* 1957, v164, 2025.

4. Mulder, J., *Lancet,* 1957, v2, 334.

5. Davenport, F. M., *Pub. Health Rep.,* 1958, v73, 133.

6. Hilleman, M. R., Flatley, F. J., Anderson, S. A., Leucking, M. L., Levinson, D. J., *J.A.M.A.,* 1958, v166, 1134.

7. Boger, W. P., Liu, O. C., *ibid.,* 1957, v165, 1687.

8. Tyrrell, D. A. J., Horsfall, F. L., Jr., *J. Immunol.,* 1952, v69, 563.

9. Hilleman, M. R., Horsfall, F. L., Jr., *ibid.,* 1952, v69, 343.

10. Archetti, I., Horsfall, F. L., Jr., *J. Exp. Med.,* 1950, v92, 441.

11. Jensen, K. E., Francis, T., Jr., *ibid.,* 1953, v98, 619.

12. Fiset, P., Depoux, R., *Bull. World Hlth. Org.,* 1954, v11, 987.

13. Hirst, G. K., *J. Exp. Med.,* 1947, v86, 357.

14. Sugg, J. Y., *J. Bact.,* 1949, v58, 399.

15. Jensen, K. E., Minuse, E., Francis, T., Jr., *J. Immunol.,* 1957, v78, 356.

16. Tamm, I., Ginsberg, H. S., Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 94.

17. Isaacs, A., Gledhill, A. W., Andrewes, C. H., *Bull. World Hlth. Org.,* 1952, v6, 287.

18. Isaacs, A., *Lancet,* 1953, v1, 676.

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Studies on Copper Metabolism. XXV. Relationship Between Serum and Liver Copper.* (24093)

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In a previous publication from this laboratory(1), 2 infants with hypochromic anemia, hypocupremia, hypoferremia and hypoalbuminemia were described. The parenteral administration of iron was followed by prompt hematologic response and by appreciable increase in serum albumin, but not by an increase in serum copper. The hypocupremia was alleviated only by administration of copper. It was suggested that hypocupremia was secondary to a deficiency of copper which was great enough to produce hypocupremia but which was not sufficiently severe to be significant in the pathogenesis of anemia. Little is known concerning the interrelationship between dietary, serum and liver copper. It has been shown in both swine(2) and rats(3) fed diets low in copper that hypocupremia precedes development of anemia. The extent to which serum copper concentrations reflect tissue concentrations of copper in animals fed diets containing low, normal and increased amounts of copper is unknown.

This paper presents observations on correlation between serum and liver copper in rats fed diets containing low, normal and increased amounts of this element. Data on blood he-

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TABLE I. Observations on Hemoglobin, Serum Copper and Serum Iron, Liver Copper and Total Serum Proteins.

Group	Day	Hemoglobin, g %	Serum copper, μg %		Wt, g	Copper, μg/g†		Serum iron, μg %‡	Total protein, g %‡
Normal copper	0		138	6.9*	8.98	3.53	.03*	161	6.8
	10		142	10.6	6.15	3.57	.23	278	
	20		135	6.2	6.58	3.73	.23	207	
	40	15.3	121	4.7	6.07	4.10	.05	222	
	60	15.5	133	6.4	5.54	3.84	.04	238	
Low copper	5		65	5.0	8.98	3.26	.07		
	10		58	3.6	7.04	2.67	.15	233	6.4
	20		44	6.6	6.80	1.58	.15	210	5.9
	40	15.5	23	4.2	6.38	2.31	.15	187	5.2
	60	14.6	24	4.4	6.14	2.08	.12	130	6.3
High Copper	0		93	2.9	6.30	4.84	.35	147	
	10		119	3.9	4.78	3.63	.35	218	
	20		132	2.7	5.44	6.38	.62	233	
	40		149	5.8	4.98	8.95	1.11	281	
	60		184	8.7	4.71	12.16	1.00	182	

* The figures refer to the mean ± stand. error of groups of 10 rats. † μg/g of wet tissue.
‡ Pooled samples.

moglobin, total serum proteins and serum iron are also reported.

Methods. One hundred and fifty male, Sprague-Dawley rats, 150 to 200 g in weight were used. The basal diet consisted of evaporated milk,‡ diluted 1:1 with demineralized water. 25 mg of ferric chloride,§ spectroscopically-free of copper, was added to 1 l of the basal diet. Since each animal consumed about 50 ml of the basal diet daily, each received 1.25 mg of iron/day. The diet fed to animals in the "normal-copper" group was supplemented with copper acetate in such an amount that each animal received 40 μg of copper daily. The diet fed animals in the "high-copper" group was supplemented with copper acetate in such an amount that each animal consumed 1.6 mg of copper daily. Ten animals in the "normal-copper" group were sacrificed on days 0, 10, 20, 40 and 60. The "low-copper" group was started on the experiment at the same time as the "normal-copper" group and 10 animals were sacrificed on days 5, 10, 20, 40 and 60. The "high-copper" group was studied at a later date and 10 rats were sacrificed on days 0, 10, 20, 40 and 60. All animals were anesthetized lightly with ether and exsanguinated *via* the abdominal aorta. The livers were removed, rinsed with copper-free water, weighed and stored in the frozen state. Methods for determination of serum copper(4), serum iron(5) and tissue copper(6) have been published previously. Total serum protein estimations were performed by the method of Weichselbaum(7). Hemoglobin was determined by the cyanmethemoglobin method(8).

Results. The data are presented in Table I and in Fig. 1. Concentration of copper in

‡ Copper concentration of 70 to 110 μg/l.

§ Prepared from carbonyl iron powder (96% iron), Grade RX, Antara Products, General Aniline and Film Corp., N.Y.City.

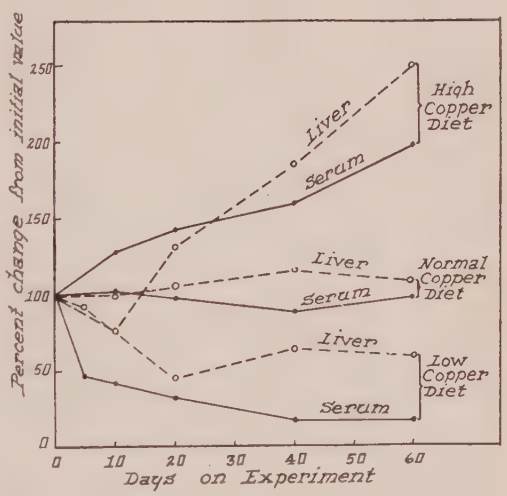

FIG. 1. Influence of dietary copper on concentration of copper in serum and liver. Values are expressed as % change from initial values.

liver and in serum of the animals in the "normal-copper" group remained relatively constant.

In the "low-copper" group, the serum copper decreased rapidly in the first 5 days to 48% of the initial value. During the next 35 days there was a more gradual decrease to a level of about 23 μg %. Thereafter, concentration remained at this low level. Concentration of copper in the liver decreased gradually over the first 20 days and thereafter remained constant at about 60% of the initial value.

In the animals fed a "high-copper" diet there was a progressive increase in concentration of copper in both the serum and the liver. Over the first 20 days there was a more prompt increase in serum copper than in liver copper. Thereafter, there was a slightly greater rate of accumulation of copper in the liver as compared with the serum (Fig. 1).

Anemia of significant degree was not present in the animals in the "low-copper" group in spite of an 80% reduction in serum copper and a 40% reduction in liver copper. The appearance of hypoferremia in the "low-copper" animals is in agreement with observations in copper-deficient pigs(2). Likewise, the failure of copper-depleted rats to develop hypoproteinemia is in agreement with observations in swine(2).

Discussion. Under conditions of these experiments, dietary intake of copper greatly influenced concentration of copper in the serum and in the liver. Within certain limits concentration of copper in the serum paralleled concentration in the liver. In general, a change in the intake of copper was reflected more promptly in the serum than in the liver. With a "high-copper" diet there was a progressive increase in the concentration in both the serum and the liver but after about 30 days there was a greater rate of accumulation of copper in the liver than in the serum. Thus, the capacity of the serum copper to increase beyond a certain point seemed more limited than was the capacity of the liver.

It is interesting that with a diet low in copper, concentration of copper in the liver decreased to about 60% of the initial value in 20 days but thereafter there was no further decrease. This suggests that there is a "labile" fraction of liver copper and a more "stable" fraction. In severely anemic copper-deficient pigs, the liver copper averages 5% of the normal values(2). Thus, the more stable fraction of liver copper is depleted in severely deficient animals with anemia. It should be pointed out that the experiments in swine were performed on rapidly growing, 5 day old pigs, whereas the rats used in the present experiments were not fed the low-copper diet until after the age when they grow most rapidly. This probably accounts for the lesser degree of depletion of liver copper in the rats than in the pigs.

It should be emphasized that concentration of copper in the serum and in the liver does not reflect dietary intake of copper under all conditions. Likewise, under certain circumstances there is no relationship between concentration of copper in the serum and in the liver. For example, in Wilson's disease(9), the dietary intake of copper is normal, concentration of copper in the serum is greatly reduced, and concentration of copper in the liver is greatly increased. In this disease there is a specific inability to synthesize ceruloplasmin, the copper protein of plasma. In conditions in which protein synthesis, rather than availability of copper, limits synthesis of ceruloplasmin(1), it may be expected that there will be disparity between concentration of copper in the serum and in the liver. Likewise, in conditions such as pregnancy and infection in which the synthesis of ceruloplasmin is accelerated(10), there may be disparity between dietary intake of copper and serum copper concentration and between concentration of copper in serum and in the liver.

Summary. 1. Dietary restriction of copper in adult rats was followed by prompt decrease in serum copper and a somewhat slower and less extensive depletion of liver copper. 2. Feeding of diets high in copper was followed by increase in concentration of copper in both serum and liver. The increase occurred more rapidly in serum than in liver during the first 20 days. Thereafter, rate of increase was greater in liver than in serum. 3. It is concluded that concentration of copper in serum and liver is markedly influenced by dietary

intake of copper and that there is a close correlation between serum and liver copper concentrations.

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1. Zipursky, A., Dempsey, H., Markowitz, H., Cartwright, G. E., Wintrobe, M. M., *A.M.A. J. Dis. Child.*, in press.

2. Lahey, M. E., Gubler, C. J., Chase, M. S., Cartwright, G. E., Wintrobe, M. M., *Blood,* 1952, v7, 1053.

3. Gubler, C. J., Lahey, M. E., Cartwright, G. E., Wintrobe, M. M., *Am. J. Physiol.,* 1952, v171, 652.

4. Gubler, C. J., Lahey, M. E., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., *J. Biol. Chem.,* 1952, v196, 209.

5. Hamilton, L. D., Gubler, C. J., Cartwright, G. E., Wintrobe, M. M., PROC. SOC. EXP. BIOL. AND MED., 1950, v65, 75.

6. Gubler, C. J., Cartwright, G. E., Wintrobe, M. M., *J. Biol. Chem.,* 1957, v224, 533.

7. Weichselbaum, T. E., *Am. J. Clin. Path., Tech. Sec.,* 1946, v16, 40.

8. Crosby, W. H., Munn, J. I., Furth, F. W., *U. S. Armed Forces Med. J.,* 1954, v5, 693.

9. Cartwright, G. E., Hodges, R. E., Gubler, C. J., Mahoney, J. P., Daum, K., Wintrobe, M. M., Bean, W. B., *J. Clin. Invest.,* 1954, v33, 1487.

10. Markowitz, H., Gubler, C. J., Mahoney, J. P., Cartwright, G. E., Wintrobe, M. M., *ibid.,* 1955, v34, 1498.

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Bile Acid Metabolism, Dietary Fats, and Plasma Cholesterol Levels.* (24094)

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The discovery of plasma cholesterol lowering properties of ingested highly unsaturated fats(1,2) has stimulated extensive study concerning the possible mechanism by which this effect is mediated. Most research has been focused upon possible changes in plasma transport or tissue disposition of cholesterol, while little attention has been directed toward possible intra-intestinal effects of ingested unsaturated fats. However, recently Gordon *et al.*(3) have reported an increased excretion of bile salts in feces of individuals ingesting sunflower seed oil (iodine No. 135) as against those ingesting hydrogenated coconut oil (iodine No. 6). Recently, we observed(4) that when absorption of bile acids (but not of cholesterol) was diminished in rats by ileal reimplantation of the common bile duct, a marked reduction in plasma cholesterol concentration occurred. In the present study the effects of ingested highly unsaturated fats upon both the flow and constituents of bile and upon absorption of cholesterol itself are reported.

Methods. A. *Acute effect of ingested highly unsaturated fats upon intestinal absorption of cholesterol.* To determine the possible effect of a highly unsaturated fat upon intestinal absorption of cholesterol, a group of 9 healthy male adult rats (Long-Evans strain) were starved for 24 hours, then given 50 mg of cholesterol dissolved in 3 ml of soy bean oil (iodine No. 121; Liebermann-Burchard positive material = 4.6 mg/ml calculated as cholesterol). Another group of 5 rats was given the same amount of cholesterol in 3 ml of corn oil (iodine No. 120; L-B positive material = 11 mg/ml). For control purposes, one group of 5 rats was given 50 mg of cholesterol in 3 ml of warmed lard (iodine No. 59; L-B positive material = 1.2 mg/ml), and another group of 5 rats, the same amount of cholesterol in 3 ml of coconut oil (iodine No. 6; L-B positive material = 1.1 mg/ml). Immediately after feeding, all rats were subjected to cannulation of intestinal lymph duct(5) and lymph collected for 24 hours was analyzed for cholesterol(6). B.

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TABLE I. Effects of Ingestion of Unsaturated Fats on Cholesterol Absorption.

	No. of rats	Avg wt (g)	Intestinal lymph (24 hr) Avg vol (ml)	Intestinal lymph (24 hr) Total cholesterol mg/100 ml	Intestinal lymph (24 hr) Total cholesterol mg/24 hr
A. Rats given soybean oil (iodine No. 121; L–B + = 4.6 mg/ml)	9	276	46.9 * 6.4	51 3.1	22.8 2.1
B. Rats given corn oil (iodine No. 120; L–B + = 11 mg/ml)	5	255	28.6 * 8.4	82 5.1	22.3 2.6
C. Rats given lard (iodine No. 59; L–B + = 1.2 mg/ml)	5	302	24.9 * 6.8	58 6.2	14.0 2.7
D. Rats given coconut oil (iodine No. 6; L–B + = 1.1 mg/ml)	5	265	25.4 * 4.8	38 4.1	8.9 3.2

* S.E. of mean.

Chronic effect of ingested unsaturated fats upon (1) Plasma cholesterol and (2) Bile cholesterol and cholate. A group of 6 rats was fed 10% suspension of walnut oil† (iodine No.‡ 131.5; L-B positive material = 3 mg/ml) for 7 days. A second group of 6 rats was fed 10% suspension of coconut oil§ (iodine No. 6; L-B positive material = 1.1 mg/ml) for 7 days. A third group of 6 rats was fed a special fat and sterol free diet.‖ Rats on the oil diets each ingested approximately 65 ml of the suspension/day. Plasma samples were obtained at beginning and end of the 7 day period and analyzed for total cholesterol. The rats then were subjected to cannulation of their bile duct(7) and the 24 hour bile collection was analyzed for its cholesterol and cholate contents according to previously described methods(8).

Results. Table I indicates almost twice as much sterol found in lymph after administration of cholesterol with either of the highly unsaturated fats as compared with that found when cholesterol is administered in either lard or coconut oil. That this sterol is cholesterol is indicated by the finding that lymph contained the same quantity of Liebermann-Burchard positive material regardless of whether cholesterol was given in soybean oil, containing 4.6 mg/ml plant sterol, or in corn oil with over twice this amount of sitosterols.

Table II indicates that as early as 7 days, continued ingestion of a highly unsaturated fat resulted in a plasma cholesterol significantly lower than in rats given either a relatively saturated fat or a diet containing neither fat nor sterol. Excretion of bile cholesterol also was significantly higher in these rats fed walnut oil. Total amount of cholate excreted by rats given walnut oil was twice as great as that observed in rats ingesting a sterol free diet and 26% greater than that excreted by rats given coconut oil.

Discussion. The present results indicate that, whatever mechanism effects a reduction in plasma cholesterol level of the animal fed highly unsaturated fats, does not involve a hindrance to intestinal absorption of sterol, because the latter is not hindered and may well be enhanced by administration of such fats.

If bile cholesterol excretion can be employed, as we think it can be, as index of hepatic rate of turnover of cholesterol(9), then it is clear that following ingestion of a highly unsaturated fat, a marked increase in cholesterol turnover occurs. This present finding confirms the similar conclusion reached by Avigan and Steinberg(10) who measured the hepatic turnover of cholesterol with radioactive carbon.

The increased rate of cholesterol turnover after administration of unsaturated fat is not followed by any increase in cholesterol content

† Walnut oil, 100 g; casein, 120 g; emulsifying agent, 3 g; NaCl, (20%), 35 ml; KCl (20%), 47 ml; distilled H_2O, 960 ml.

‡ Determined by Dr. George D. Michaels.

§ Exactly similar to † except 100 g of coconut oil substituted for walnut oil.

‖ Cane sugar, 7100 g; Na Caseinate, 2,500 g; NaCl, 150 g; Salts W., 200 g (Nutritional Biochemicals Corp.) Vit. mixture "Litrison" (R) (Hoffmann La-Roche).

TABLE II. Effect of Ingestion of Unsaturated and Saturated Fats upon Plasma Cholesterol and Bile Cholesterol and Cholate.

No. of rats	Avg wt			Plasma cholesterol (mg/100 ml)			Bile†				
								Total cholesterol		Total cholate	
	Beg.	7 days	21 days	Beg.	7 days	21 days	Vol	mg/100 ml	mg/24 hr	mg/100 ml	mg/24 hr
				Walnut oil (iodine No. 131.5; L–B + = 3 mg/ml)							
6	276	237	—	56	43	—	11.8	17.7	2.1	459	58
				* 3.1	2.0				.16		3.7
				Coconut oil (iodine No. 6; L–B + = 1.1 mg/ml)							
6	260	224	—	60	62	—	10.1	16.3	1.6	436	43
				* 2.4	4.2				.05		2.5
				Sterol-free diet							
6	272	259	—	58	52		12.2	9.6	1.2	331	25
				* 3.2	1.7				.17		2.1

* S.E. of mean. † Bile collected for 24 hr, after 7 days on respective diets.

of plasma(11) and indeed, not always followed even by increase in content of cholesterol in the liver(12). This of course suggests that the newly formed cholesterol is being converted further. In view of the fact that the principal end product of cholesterol metabolism in the body is bile acid(13), the implication is strong that the liver of the animal fed highly unsaturated fats is producing an increased amount of bile acids. This possibility is greatly strengthened by the present findings of an increased biliary excretion of bile acids after feeding of walnut oil and by the recent observation of Gordon *et al.*(3) that there is an increased fecal excretion of bile acids in persons fed sunflower seed oil. This last observation suggests either that an excess of bile acid is being excreted or that less is being reabsorbed. It therefore seems likely that enhanced removal of bile acid from the body provokes increased cholesterol turnover. This suggestion is reinforced by the finding of Beher and Baker(14) that *feeding* of cholic acid greatly *reduces* rates of synthesis and mobilization of cholesterol in the rat.

In this connection it is of interest that when an animal is induced to excrete more bile acid as occurs in the hyperthyroid state(15) or when bile acid reabsorption is interfered with (4), plasma cholesterol promptly falls. It is our suggestion that a similar mechanism is in play in the observed reduction in plasma cholesterol of the subject ingesting highly unsaturated fats.

Summary. 1. Administration of highly unsaturated fats to the rat effected a greater intestinal absorption of cholesterol than similar administration of saturated fats. 2. Following continued ingestion of highly unsaturated fat, there was evidence that the fall in plasma cholesterol observed was accompanied by a marked increase in excretion of both cholesterol and bile acid in the bile. 3. The possible relationship of reduction in plasma cholesterol to the observed change in bile cholesterol and bile acid excretion is discussed.

1. Kinsell, L. W., Michaels, G. D., Partridge, J. W., Boling, L. A., Balch, H. E., Cochrane, G. C., *J. Clin. Nutrition,* 1953, v1, 224.

2. Ahrens, E. H., Jr., Isaltas, T. T., Hirsch, J., Insull, W., Jr., *J. Clin. Invest.,* 1955, v34, 918.

3. Gordon, H., Lewis, B., Eales, L., Brock, J. F., *Nature,* 1957, v180, 923.

4. Byers, S. O., Friedman, M., *Am. J. Physiol.,* 1958, v192, 427.

5. Friedman, M., Byers, S. O., Shibata, E., *J. Exp. Med.,* 1953, v98, 107.

6. Saifer, A., Kammerer, O. F., *J. Biol. Chem.,* 1946, v164, 657.

7. Friedman, M., Byers, S. O., Michaelis, F., *Am. J. Physiol.,* 1950, v162, 575.

8. ———, *ibid.,* 1951, v164, 786.

9. Byers, S. O., Friedman, M., *ibid.,* 1952, v168, 297.

10. Avigan, J., Steinberg, D., *Circulation,* 1957, v16, 492.

11. Hegsted, D. M., Gotsis, A., Stare, F. J., *ibid.,* 1957, v16, 479.

12. Diller, E. R., Woods, B. L., Harvey, O. A., *ibid.,* 1957, v16, 505.

13. Friedman, M., Byers, S. O., Gunning, B., *Am. J. Physiol.,* 1953, v172, 309.

14. Beher, W. T., Baker, G. D., *Abst. Am. Chem. Soc.*, San Francisco, p22C, April 13, 1958.
15. Rosenman, R. H., Byers, S. O., Friedman, M., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 1287.

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Incorporation and Conversion of Lysine-2-C^{14} in Rat Biopsy-Connective Tissue. (24095)

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The amino acids, hydroxyproline and hydroxylysine, are found chiefly in collagen and are responsible in part for stability of the fiber(1,2). Hydroxyproline cannot be incorporated directly into the peptide chain of collagen, but a portion of the proline becomes hydroxylated following its incorporation(3). Presumably lysine and hydroxylysine are involved in collagen formation in a similar manner because only labeled lysine fed to an animal is incorporated into collagen and a portion appears as hydroxylysine(4).

The present report deals with incorporation, distribution and rate of turnover of C^{14}-labeled lysine in rat connective tissue obtained by sponge implantation technic(5). Conversion of labeled lysine to hydroxylysine in connective tissue of different ages and from both sexes is also presented.

Methods. Sprague-Dawley rats of approximately 6 months of age and weighing 200 to 250 g were used. All animals included were given a single intraperitoneal injection of 1 ml of neutralized dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc) in physiological saline. Three polyvinyl sponges (Ivalon†) were implanted subcutaneously along dorsum of rat. Previous histological work indicates growth of normal appearing connective tissue into the interstices of the sponge(5). The fact that rate of collagen formation in sponge biopsy(6) was similar to that found by Abercrombie *et al.* in wound healing in the rat(7) suggests comparable metabolic rates for the 2 connective tissues. Incorporation of labeled lysine and formation of tagged hydroxylysine were studied in connective tissue of 2 male and 2 female rats, 21 days after sponge implantation and in one male and one female rat 300 days after implantation. The tissues were removed 16 hours following intraperitoneal injection. Sponge-tissues were weighed, homogenized with 10 ml of physiological saline in VirTis "45" Macro Homogenizer‡ for 5 minutes with rheostat reading at 50, and centrifuged at approximately 15,000 x g at 5°C for 1 hour to obtain the saline-soluble protein. The residue was washed with distilled water and centrifuged at approximately 15,000 x g at 5°C for 30 minutes, and the wash discarded. The resultant residue was shaken with 5 ml of 0.5 M acetic acid at 5°C for three 24-hour periods in 12 ml plastic tube with rubber stopper. After each 24-hour period, the plastic tubes were centrifuged at 90,000 x g in a Spinco Model E ultracentrifuge for 1 hour and the acetic acid-soluble protein decanted. The 3 acetic acid extracts were combined. Protein of the saline-soluble fraction was precipitated with 4 volumes of 10% trichloroacetic acid. The trichloroacetic acid precipitate of the saline, the acetic acid-soluble protein and the residue were hydrolyzed with 6 N HCl in sealed pyrex tubes at 110°C for 16 hours. Nitrogen was determined by Conway microdiffusion technic(8) and converted to a protein value by factor 6.25. An aliquot of the hydrolysate was evaporated to dryness on steam bath to remove excess HCl, neutralized and again evaporated to dryness. The neutralized samples were dissolved in citrate buffer, pH 5.0, to concentration of 15-20 mg

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of protein/ml, and 1 ml was applied to a 15 cm column of Dowex 50-x 8 (200-400 mesh). The method of Moore and Stein(9) for basic amino acid chromatography was used. The column was eluted first with 30 ml of citrate buffer, pH 5.0, and this eluate was discarded. A phosphate buffer, pH 6.8, was then used, and the eluate was collected in 2 ml fractions. An aliquot of each fraction was placed in aluminum planchet and evaporated to dryness for radioactivity measurement, and an aliquot was used for the ninhydrin reaction(10). The effects of age of tissue and of sex of donor rat upon incorporation of lysine and synthesis of hydroxylysine were studied in 17-, 50- and 300-day old tissues from female rat and in 17-, 90- and 300-day old tissues of the male, which were removed 16 hours after lysine injection. Protein of connective tissue biopsies was partitioned into saline-soluble and -insoluble fractions as previously described. The saline-insoluble residue was then divided into collagen and non-collagenous protein by the method of Fitch, who reported 95 to 100% extraction of collagen with hot (90°C) trichloroacetic acid extraction(11). Similar extraction results were found from the sponge-connective tissue. All fractions were hydrolyzed with 6 N HCl in sealed pyrex tubes for 16 hours at 110°C, and neutralized aliquots of the hydrolysates were taken for column chromatography and analysis as described. Turnover rates of lysine- and hydroxylysine-containing proteins of biopsy-connective tissue were followed in a female rat and in 2 male rats. The animals, which, at age of 4 to 6 months, had been previously implanted with 2 or 3 sponges, were given intraperitoneal injection of labeled lysine, and connective tissue biopsies were removed at different intervals of time following injection. The connective tissue-sponges in the female, which were 50 days of age at time of injection, were removed at 8 hours, 17 and 31 days following lysine injection. Tissues in male rats were 90 days old at time of injection and were removed after 8 hours and 11 days. The tissues were fractionated into saline-soluble protein, collagen and non-collagenous protein. Nitrogen and radioactivity were determined on neutralized acid hydrolysates of tissue fractions, and the specific activity (total cpm/mg of protein) was calculated.

TABLE I. Lysine and Hydroxylysine in Rat Biopsy-Connective Tissue.

	Male		Female	
	21-day*	300-day	21-day*	300-day
Amino acid conc. (% of nitrogen)				
Hydroxylysine				
Saline-soluble protein	0	0	0	0
Acetic acid-soluble protein	.36	.35	0	.94
Residue	.60	.85	.62	.60
Lysine				
Saline-soluble protein	7.43	8.90	9.82	8.41
Acetic acid-soluble protein	5.53	7.54	6.79	5.10
Residue	5.16	4.76	4.72	4.99
Specific activity (cpm/mg of amino acid)				
Hydroxylysine				
Acetic acid-soluble protein	940	264		322
Residue	541	246	990	446
Lysine				
Saline-soluble protein	953	878	849	897
Acetic acid-soluble protein	1180	629	892	850
Residue	1073	282	1182	1278

* Mean of 2 values.

Tissues were removed 16 hr after inj. of dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc).

Results. Concentrations and specific activities of lysine and hydroxylysine in saline-soluble protein, acetic acid-soluble protein and the residue of biopsy-connective tissue 16 hours after injection of lysine-2-C^{14} are shown in Table I. Hydroxylysine was not found in the saline-soluble protein of biopsy-connective tissue. In male biopsy-connective tissue, hydroxylysine concentration was lower in the acetic acid-soluble protein than in the residue and was lower in the residue of 21-day than in that of 300-day old tissue. Hydroxylysine was apparently not present in the acetic acid-soluble protein of the tissue from the female at 21 days but was present in this fraction in the tissue of 300 days of age. Hydroxylysine concentrations in the acetic acid-insoluble residue of female biopsy-connective tissue of 21 and 300 days of age were not different. Ly-

TABLE II. Incorporation and Conversion of Lysine-2-C^{14} in Biopsy-Connective Tissue.

	Lysine		Hydroxylysine	
	%*	S.A.†	%	S.A.
17-day tissue				
Collagen				
♂	3.22	858	.67	939
♀	3.09	784	1.02	804
Non-collagen protein				
♂	9.73	739	.0	0
♀	7.84	1074	.0	0
50-day female tissue				
Collagen	3.67	485	.94	393
Non-collagen protein	10.12	1211	.0	0
90-day male tissue				
Collagen	3.47	332	.81	303
Non-collagen protein	11.46	1007	.0	0
300-day tissue				
Collagen				
♂				246‡
♀				446‡

* Grams of amino acid/100 g of protein.
† S.A. = Specific activity (cpm/mg amino acid).
‡ Data from acetic acid fractionation. Since hydroxylysine exists only in collagen fraction, these data, calculated on basis of hydroxylysine concentration, are used for comparison.

sine concentration was greatest in the saline-soluble fraction and lowest in the residue of the 21-day and 300-day old tissues of both sexes.

Specific activities of hydroxylysine (cpm/mg of amino acid) in the acetic acid-soluble protein and residue were lower in the 300-day old tissue than in the 21-day old tissue in both sexes, with the exception of the acetic acid-soluble protein of female tissue in which there was no measurable amount for comparison at 21 days of tissue age. Specific activity of lysine did not decrease with tissue age in any fraction of female tissue, but in the male there was a reduction in activity of both fractions of saline-insoluble residue of tissue from 21 to 300 days of age.

The acetic acid-soluble and -insoluble fractions consist of collagen and a non-collagenous protein(6). Determination of specific activity of lysine and of hydroxylysine in these 2 proteins of tissue, which were separated by hot trichloroacetic acid extraction of the collagen from saline-insoluble residue of male and female connective tissue, indicated that hydroxylysine was found entirely in the collagen. The labeled lysine was found in the collagen, the non-collagenous protein and in saline-soluble protein. Of the total protein of tissue, 36.4% was in the collagen, 21.8% in the non-collagenous fraction and 41.8% in the saline-soluble fraction.

The effects of tissue age and of sex of donor rat upon incorporation of lysine-2-C^{14} and its conversion to labeled hydroxylysine in biopsy-connective tissue are shown in Table II. In the collagen, specific activity of both lysine and hydroxylysine decreased with increase in tissue age from 17 to 50 days in the female and from 17 to 90 days in the male. This decrease appeared to be more marked in the male. Specific activity of hydroxylysine of the 300-day old female tissue was twice that of similarly aged male tissue. There was no parallel reduction in specific activity of lysine with tissue age in the non-collagenous protein in tissue of either sex.

Concentrations and radioactivities of the basic amino acids of saline-soluble protein, acetic acid-soluble protein and residue are shown in Fig. 1. Only the basic amino acids contained radioactivity. Radioactivity in the saline-soluble protein was confined to lysine. The acetic acid-soluble protein and the residue contained tyrosine, phenylalanine, histidine, hydroxylysine and lysine.

The composite radioactivity decay curves for saline-soluble, collagen and non-collagenous fractions of tissues obtained from male and female rats are plotted as specific activity (total cpm/mg of protein) against time in days in Fig. 2. Radioactivity in non-collagenous and saline-soluble fractions rose promptly during the first 8 hours and decayed during the following 10 days to less than 40% of the 8-hour value. Rate of decay then slowed so that the activity at 30 days was approximately 10% of maximum. Maximum activity of the collagen was reached within 8 hours following injection of the isotope but rose to only 30% of maximum specific activity of the non-collagenous protein. Decay of activity in the collagen occurred more slowly than in the other 2 fractions, and at 30 days, 30% of the activity remained.

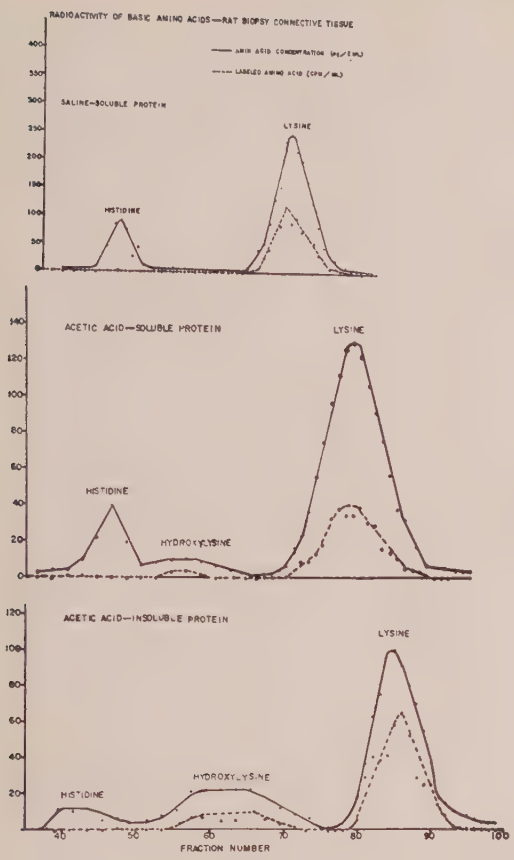

FIG. 1.

Discussion. Incorporation of lysine-2-C^{14} into sponge-biopsy connective tissue and conversion of a portion of tagged lysine to hydroxylysine has been demonstrated. Labeled lysine administered intraperitoneally becomes incorporated within 8 hours into protein of ground substance (saline-soluble fraction), into non-collagenous protein and into collagen. In recent experiments not included in this report labeled lysine and hydroxylysine were found in the collagen within 3 hours following intraperitoneal injection.

Rapid incorporation of labeled lysine into connective tissue does not indicate a rapid turnover since in the collagen the loss of radioactivity is slow. However, metabolic activity of sponge-connective tissue would appear to be greater than that of tendon(12) since tagged hydroxylysine appeared in young collagen within 8 hours following injection of labeled lysine and only 30% of the radioactivity remained after 30 days. Specific activities of lysine in the acetic acid-soluble and -insoluble proteins of the 21-day old tissue (Table I) were greater than in the hot trichloroacetic acid-soluble collagen of the 17-day old tissue (Table II), and the difference is unquestionably related to metabolically active non-collagenous protein present in the 2 acetic acid fractions. The relatively rapid disappearance of radioactivity from the saline-soluble protein and the non-collagenous protein of the residue of connective tissue indicates a rapid turnover of these proteins of ground substance, the cellular protoplasm and the non-collagenous protein.

Incorporation of labeled lysine in the collagen fraction and a conversion of a portion to hydroxylysine are approximately the same for both sexes at 17 days of tissue age with, perhaps, the tissue of the male being somewhat more active than the female. Collagen of the 50-day old tissue from the female rat has approximately 50% of specific activities of lysine and hydroxylysine found in collagen from the 17-day old tissue. In the collagen iso-

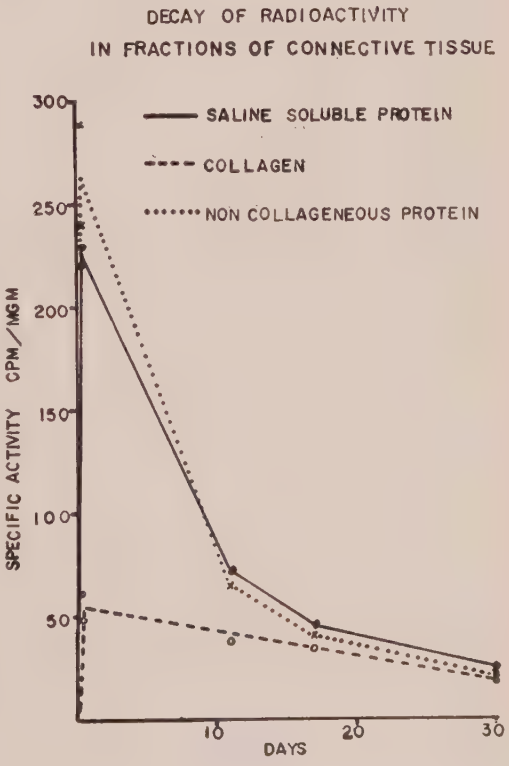

FIG. 2.

lated from the 90-day old tissue of the male rat there is a similar reduction. Specific activity of hydroxylysine in collagen of the 300-day old tissue of the male is 26% that of 17-day old tissue, while in the 300-day old tissue of the female, 55% of the 17-day tissue value was observed. This would seem to indicate that the female connective tissue has as active an hydroxylating mechanism at 300 days as found at 50 days of tissue age. It would appear that the tissue of the male has a reduction in rate of hydroxylation with tissue age. Yet it has been demonstrated that no increase in collagen content occurs after 21 days of tissue growth in the female rat, while the male tissue continues to accumulate collagen to 300 days of tissue age(6). This suggests that either rate of conversion of lysine to hydroxylysine is not an index of collagen formation or that the female tissue contains some substance which enhances breakdown of collagen at the same rate as its synthesis.

Summary. 1. C^{14}-labeled lysine administered intraperitoneally into the rat is incorporated within hours into the saline-soluble and -insoluble non-collagenous protein and collagen of biopsy-connective tissue. Radioactivity is confined to lysine in all fractions of connective tissue except for a portion which is converted to hydroxylysine in the collagen. 2. Following appearance of radioactivity in all fractions of connective tissue, decay occurs rapidly in saline-insoluble non-collagenous protein and saline-soluble protein and slowly in the collagen fraction. Thirty days after injection of labeled lysine approximately 10% of maximum activity remained in the saline protein fractions while 30% remained in collagen. 3. Incorporation of labeled lysine into the non-collagenous protein of connective tissue is not influenced by tissue age while its incorporation into the collagen appears to decrease with tissue age. 4. Specific activity of labeled hydroxylysine from a 300-day old tissue of a female rat was approximately twice that of a comparably aged tissue from a male rat.

1. Gustavson, K. H., *Svensk Kem Tidskr.,* 1953, v65, 70.

2. ———, *The chemistry and reactivity of collagen,* Academic Press, 1956, 135.

3. Stetten, M. R., Schoenheimer, R. J., *J. Biol. Chem.,* 1944, v153, 113.

4. Sinex, F. M., Van Slyke, D. D., *ibid.,* 1955, v216, 245.

5. Boucek, R. J., Noble, N. L., *A.M.A. Arch. Path.,* 1955, v59, 553.

6. Kao, K. Y. T., Boucek, R. J., Noble, N. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 535.

7. Abercrombie, M., James, D. W., *J. Embryol. Exp. Morph.,* 1957, v5, 171.

8. Conway, E. J., *Microdiffusion analysis and volumetric error,* D. Van Nostrand Co., 1950, 3rd Ed., 124.

9. Moore, S., and Stein, W. H., *J. Biol. Chem.,* 1951, v192, 663.

10. ———, *ibid.,* 1954, v211, 907.

11. Fitch, S. M., *Nature,* 1955, v176, 163.

12. Neuberger, A., Perrone, J. C., Slack, H. G. B., *Biochem. J.,* 1951, v49, 199.

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In vitro Incorporation and Hydroxylation of Lysine-2-C^{14} In Rat Biopsy-Connective Tissue.* (24096)

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Labeled lysine administered orally or intraperitoneally to the rat appears in the collagen fraction of connective tissue as lysine and hydroxylysine(1,2). The purpose of the present study was to determine the *in vitro* incorporation and hydroxylation of lysine-2-C^{14} in con-

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nective tissue obtained by the sponge biopsy technic(3) and to compare results with those obtained from *in vivo* studies. The study of hydroxylation of lysine *in vitro* provides an adaptable technic for study of biosynthesis of collagen.

Methods. Three polyvinyl sponges (Ivalon‡) were implanted subcutaneously along lower dorsum of 6-month-old Sprague-Dawley rats by a previously described technic(3). At different intervals of tissue age, the sponge-connective tissue was removed. *In vitro* studies. The sponges were removed from a male and a female rat 18 days following implantation, from a female rat after 24 days and from 2 male rats after 34 days. The sponge-connective tissue was immediately placed into ice-cold Krebs-Henseleit solution (4), which had been gassed 30 minutes with 95% O_2:5% CO_2. The tissue was kept chilled during preparation for incubation. The capsule was removed and sponge-connective tissue was sliced as thin as possible with razor blade. Approximately 500 mg of tissue was placed in 25 ml Erlenmeyer flask containing 5 ml of Krebs-Henseleit solution. One ml of neutralized solution of lysine-2-C^{14} in physiologic saline (0.255 mg dl-lysine-2-C^{14} monohydrochloride or 1 μc) was added to each flask. After the flasks were gassed with 95% O_2:5% CO_2 for one minute and capped with a rubber stopper, they were incubated, with constant agitation, in 37°C water bath. At 2-hour intervals, the flasks were removed and gassed. To study rate of lysine incorporation, flasks containing slices of 34-day-old tissue were removed in duplicate from water bath after 0, 1, 2, 4, 6, 8, and 12 hours incubation and immediately placed in freezer at –28°C. Other tissues were incubated 12 hours. To determine the effect of a complete amino acid mixture upon conversion of lysine to hydroxylysine, tissue culture medium #199§ without bicarbonate(5) was used as incubation medium instead of Krebs-Henseleit solution. Slices of two 24-day-old tissues from a female rat were equally divided among 6 incubation flasks, 3 containing 5 ml of Krebs-Henseleit solution and 3 containing 5 ml of #199 medium. One ml of neutralized lysine-2-C^{14} was added to each flask, and the flasks incubated 12 hours. *In vivo* studies. Sponge-connective tissue was obtained from 2 male rats 90 days after sponge implantation, after 17 days of connective tissue growth from a male and a female rat and after 50 days growth in female rat. All animals received a single intraperitoneal injection of one ml of neutralized dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc) in physiological saline. The rats were sacrificed and tissues removed 8 hours following injection. *Tissue analysis.* Tissues were removed from incubation mixture and blotted to remove excess lysine-2-C^{14}. The blotted tissue slices and tissues removed in the *in vivo* experiments were prepared for analysis by homogenizing in physiologic saline containing 0.25% lysine in a VirTis "45" macro homogenizer‖ for 5 minutes with rheostat reading at 50. The homogenates were centrifuged at 15,000 x g for one hour. The saline-soluble protein of supernatant was precipitated with 4 volumes of 10% trichloroacetic acid (TCA). The saline-insoluble residue was washed once with physiologic saline and again centrifuged at 15,000 x g. The supernatant was discarded and the residue separated into collagen and non-collagenous protein by the hot 0.3 M TCA extraction technic of Fitch *et al.*(6). The precipitated protein of the saline-soluble fraction, collagen and non-collagenous protein were hydrolyzed with 6 N HCl at 110°C for 16 hours in sealed pyrex tubes. Nitrogen was determined by Conway microdiffusion method(7) and converted to protein value by the 6.25 factor. Hydrolysates were evaporated on water bath to remove excess hydrochloric acid, neutralized and made up to volume. One ml of neutralized hydrolysate was evaporated to dryness on aluminum planchet for determination of radioactivity of the protein fraction. The remainder of the hydrolysate was evaporated to dryness and redissolved in citrate buffer pH 5.0 and placed on a 15 cm column of Dowex 50-x8 according to the method of Moore and

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§ Available from Microbiological Associates, Bethesda, Md.

‖ VirTis Co., Yonkers, N. Y.

TABLE I. Rate of Incorporation of Lysine-2-C^{14} in Rat Biopsy-Connective Tissue.

Sex	Age of tissue (days)	Time of incubation (hr)	Specific activity (cpm/mg of protein): Saline-soluble protein	Saline-insoluble residue: Collagen	Saline-insoluble residue: Non-collagenous protein
♂	34	0	54	26	5
		1	808*	42	431
		2		73	923
		4	1,827*	76	—
		6		176	2,000
		8	4,650	216	2,875
		12	3,020	426	4,705
♂	34	0	71	24	44
		1	528*	68	396
		2		75	678
		4	1,892*	149	1,910
		6		143	1,970
		8	3,336	370	3,735
		12	3,202	683	3,123
♀	24	12	4,465	421	4,203
♂	18	12	—	460	4,703
♀	18	12	—	409	3,376

* Combined samples. —, not done.

Stein for basic amino acid chromatography (8). Aliquots from each fraction of the eluate were dried on aluminum planchets for measurement of radioactivity. Specific activity of lysine and hydroxylysine was calculated on the basis of ninhydrin reaction (9) of aliquots of each eluate fraction.

Results. Rate of incorporation of lysine-2-C^{14} into biopsy-connective tissue is shown in Table I. Specific activities of saline-soluble protein and of non-collagenous protein increased with length of incubation and plateaued in most tissues after 8 hours of incubation. Specific activity of collagen appeared to increase to 12 hours of incubation. Incorporation rate of younger tissue, *i.e.*, 18 days and 24 days, seemed to approximate that of 34-day-old tissue.

Chromatographic separation of the basic amino acids indicated that lysine-2-C^{14} was incorporated into lysine of the saline-soluble protein, of collagen and of non-collagenous protein of biopsy-connective tissue and was converted to labeled hydroxylysine in the collagen fraction. These findings for the *in vitro* system are similar in this respect to the results of the *in vivo* study (2).

Comparison of *in vitro* and *in vivo* study of incorporation and hydroxylation of lysine-2-C^{14} is made in Table II. Specific activities of the 3 protein fractions of tissues of the *in vitro* study were greater than those of tissues of the *in vivo* study. Specific activity of lysine in collagen isolated from tissues studied *in vitro* was approximately 10 times greater than that of collagen from older tissue (50 and 90 days of age) and 5 times that of young tissue (17 days of age) studied *in vivo*. Conversion of labeled lysine to hydroxylysine occurred in incubated tissue slices although a relatively smaller amount of lysine incorporated into collagen was hydroxylated when compared with injected rats. In *in vivo* studies, there

TABLE II. Comparison of *In Vitro* Study with *In Vivo* Study of Incorporation and Hydroxylation of Lysine-2-C^{14} in Biopsy-Connective Tissue.

	Sex & No. of tissues	Age of tissue (days)	Specific activity (cpm/mg protein or amino acid): Saline-soluble protein (Protein)	Saline-insoluble protein: Collagen (Protein)	Collagen (Lysine)	Collagen (Hydroxylysine)	Saline-insoluble protein: Non-collagenous protein (Protein)
In vitro (12-hr incubation)	♂ 1	34	3,020	426	4,722	1,679	4,705
	♂ 1	34	3,202	683	4,601	1,148	3,123
	♀ 1	24	4,465	421	3,827	1,779	4,203
In vivo (8 hr after inj.)	♂ 2	90	230	48	335	310	249
	♀ 1	50	216	62	487	397	266
	♂ 1	17	151	64	858	939	233
	♂ 1	17	139	74	784	804	166

TABLE III. Incorporation of Lysine-2-C^{14} in Krebs-Henseleit Solution and in Tissue Culture Medium #199.

	Specific activity (cpm/mg protein)		
		Saline-insoluble residue	
	Saline-soluble protein	Collagen	Non-collagenous protein
Krebs-Henseleit solution	4,465	425 ± 197*	4,203 ± 699
#199 medium	1,570	127 ± 34	1,661 ± 165

* Mean and stand. dev. of 3 tissues.

was a 1:1 ratio of specific activity of labeled lysine to that of tagged hydroxylysine in collagen while in collagen of the *in vitro* study a ratio of 4:1 existed.

Comparison of incorporation and hydroxylation of lysine-2-C^{14} in tissue slices incubated in Krebs-Henseleit solution and in tissue culture medium #199 is made in Table III. Specific activities of protein fractions of tissues incubated in Krebs-Henseleit medium are significantly higher than those in the #199 medium.

Discussion. Labeled lysine can be demonstrated to be incorporated *in vitro* into tissue proteins of preformed connective tissue. Labeled lysine not only becomes incorporated into the collagen fraction but also is hydroxylated and appears as labeled hydroxylysine. *In vitro* formation of hydroxylysine indicates a biochemical process beyond incorporation or attachment of an amino acid, such as lysine, as an end group of a protein molecule and suggests the formation of at least a peptide which becomes an integral portion of collagen since hydroxylysine is found only in collagen.

Specific activities of the saline-soluble and non-collagenous protein increased with incubation time up to 8 hours while the lower specific activity of collagen increased to 12 hours. Incubation was not continued beyond 12 hours because of the possibility of growth of bacterial contaminants. The fact that there is an increase in radioactivity with time in the protein fractions further suggests that incorporation of the labeled lysine involves a metabolic process and is not a mere attachment through an epsilon linkage to a protein molecule.

The much greater specific activity of lysine in tissues studied *in vitro* than that of *in vivo* studies may be related in part to the larger concentration of labeled amino acid presented to tissue in the incubation flask. If the specific activity or weight of the injected labeled lysine were related to the approximate protein content of a 200 g rat, about one-eighth as much radioactive lysine was available to tissue of the intact rat as was placed in the incubation flask. In addition, labeled lysine administered to the intact animal would be further diluted by inactive lysine of the body.

Comparison of incorporation of labeled lysine into connective tissue incubated in Krebs-Henseleit with that incubated in a synthetic amino acid mixture (#199), indicates a significantly greater incorporation in Krebs-Henseleit medium. Since the synthetic amino acid mixture contained 7 mg % of unlabeled lysine (*i.e.*, 0.35 mg/5 ml of #199 medium), this finding is not surprising because dilution of the labeled amino acid and competition, such as occurred in *in vivo* experiments, by all proteins for labeled and non-labeled lysine would no doubt occur. The uniform depression of lysine incorporation into the saline-soluble and non-collagenous proteins as well as into collagen suggests a lack of augmentation of collagen synthesis by a commonly used and supposedly more ideal medium for tissue culture work, *i.e.*, tissue culture medium #199.

Specific activity of collagen from tissue of the *in vivo* experiments was a greater percentage of total specific activity of the 3 protein fractions than was that of tissues of the *in vitro* study. Specific activity of collagen of the *in vivo* experiments was approximately 20 to 50% that found either in saline-soluble or in non-collagenous protein, while collagen from *in vitro* studies was approximately 10% that found in either of the other 2 protein fractions. Specific activity of lysine in collagen of the *in vitro* study was 3 to 4 times higher than that of the hydroxylysine, while specific activity of lysine of collagen of the *in vivo* study was about equal to that of the hydroxylysine. These findings suggest that while collagen of *in vivo* studies had a lower specific activity than that of *in vitro* experiments, the former system converts lysine to hydroxylysine more effectively and thus pro-

vides an index of extent of collagen formation.

Summary. 1. Lysine-2-C^{14} becomes incorporated and hydroxylated in connective tissue incubated in Krebs-Henseleit medium for 12 hours. 2. Specific activity of collagenous and non-collagenous protein in the tissue studied *in vitro* was greater than that in tissue studied *in vivo*. 3. Hydroxylation of labeled lysine appears to occur more effectively in connective tissue of rats given labeled lysine intraperitoneally than in the connective tissue incubated with lysine-2-C^{14}.

1. Sinex, F. M., Van Slyke, D. D., *J. Biol. Chem.*, 1955, v216, 245.

2. Kao, K.-Y. T., Boucek, R. J., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, in press.

3. Boucek, R. J., Noble, N. L., *A.M.A. Arch. Path.*, 1955, v59, 553.

4. Grossman, C. M., Hanschildt, J. D., *J. Clin. Invest.*, 1952, v31, 192.

5. Morgan, J. F., Morton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

6. Fitch, S. M., Harkness, M., Harkness, R. D., *Nature*, 1955, v176, 163.

7. Conway, E. J., *Microdiffusion Analysis and Volumetric Error*, 3rd Ed., D. Van Nostrand Co., London, 1950, 124.

8. Moore, S., Stein, W. H., *J. Biol. Chem.*, 1951, v192, 663.

9. ———, *ibid.*, 1954, v211, 907.

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Elevated Vitamin Levels in Cerebrospinal Fluid in Multiple Sclerosis.* (24097)

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Very few studies have been reported on vitamin content of CSF (cerebrospinal fluid) in health and in disease. Since vitamins supply the prosthetic groups of enzymes, observations of their absence, or inability of absorption or transmission to the proper tissues may be forged into tools to elucidate specific metabolic dysfunctions; vice versa their pathological accumulation may be correlated with hyperfunction(1). Cyanocobalamin and folic acid (PGA) are known to provide the enzymatic apparatus for important steps in the synthesis of nucleic acids. Disturbances in distribution of these vitamins may interfere with the transfer of methyl groups and upset the equilibrium between ethanolamine and choline, or between the respective phosphatides cephalin and lecithin(2). These chemical changes in turn may give rise to demyelination, a histological picture of obscure, but undoubtedly enzymatic etiology(3).

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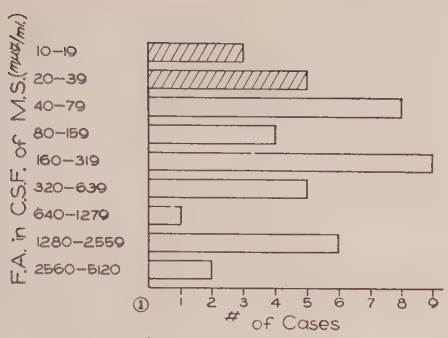
Part of results were presented at Internat. Congress of Neurological Sciences, Brussels, Aug. 1957.

Methods. The quantities of these vitamins in body fluids, tissues and organs are of such minute order of magnitude that conventional methods of chemical analysis cannot be used, but one has to resort to microbiological assay (4). The analysis of PGA offers particular intricacies, since PGA occurs conjugated to variable extent with peptides of variable molecular weight, requiring enzymatic deconjugation previous to microbioassay; the enzymatic material which must be introduced is itself a source of folic acid which considerably complicates the analysis. We have described (5) a microbioassay, using a thermophilic bacillus which grows rapidly on a simple, purely synthetic medium and which responds equally well to various conjugates as to free folic acid, thus obviating previous enzymatic deconjugation. In the case of Vit. B_{12} preference has hitherto been given to the flagellate *Euglena gracilis* over various strains of *Lactobacillus* used for this purpose, because of its higher sensitivity and specificity(6). However, even *Euglena* shows a wider spectrum than appears desirable, as it responds to certain congeners of Vit. B_{12}, which are ineffective in man and



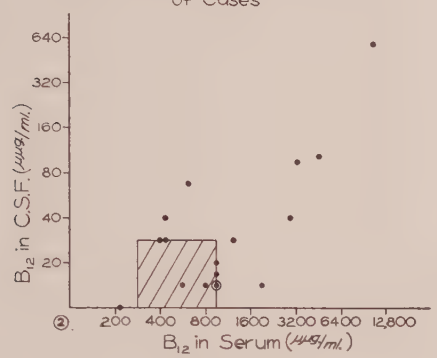


FIG. 1. Folic acid levels in CSF of 43 cases of multiple sclerosis. Normal range 10-30 mμg, hatched staves.

FIG. 2. B_{12} levels in CSF and serum in 20 cases of multiple sclerosis. Normal level in serum 300-1000 μμg/ml. Normal level in CSF 0-30 μμg/ml. Hatched area.

higher animals. We therefore use a test with *Ochromonas malhamensis,* which has been reported to cover those forms of B_{12} to which higher animals respond(7).

Results. We first established the levels of B_{12} and PGA in normal CSF, obtained from a number of non-neurological cases, prior to operative procedures. The normal range of folic acid in CSF is 10-30 mμg/ml; PGA appears to be evenly distributed between serum and CSF.

The figures for B_{12} are 0-30 μμg/ml in CSF as against 300-1000 μμg/ml in blood serum. Vit. B_{12} is known to pass the glomerular membrane without much hindrance, but its rather elevated molecular weight causes considerable difficulty in crossing the blood-brain barrier.

Comparison of vitamin levels in 310 unselected neurological cases shows an unexpectedly high incidence of values above the range observed in normal controls. The patients in our series did not receive vitamin medication. Deviations are found in PGA level of CSF and in B_{12} level of both serum and CSF. Their incidence appears to be concentrated in patients with multiple sclerosis, (MS) comprising a group of 43 cases.

Here PGA was found elevated in CSF in 34 out of 42 cases tested (Fig. 1). The levels of B_{12} in CSF were elevated in 14 out of 41 cases. Amongst these, 9 cases were higher than twice the upper limit of the normal range. The B_{12} values in blood serum of MS patients show a similar incidence of elevated values with 6 out of 20 cases above 1000 μμg/ml and 7 additional cases at 1000 μμg/ml, which is considered the upper limit of the normal range for this vitamin. The values for B_{12} in CSF and serum show fair correlation (Fig. 2).

Discussion. In pernicious anemia administration of folic acid initially benefits the patient clinically, but it seems to mobilize all available stores of B_{12}, primarily in liver and possibly in bone marrow; thus depletion of the organism of B_{12} after a few weeks gives rise to severe neurological symptoms (subacute combined degeneration of the cord). No analogy to this functional antagonism between the 2 vitamins exists in MS. No correlation was found between high PGA and low B_{12} values. In fact 4 cases among the highest B_{12} values in serum and CSF are ranking high among the folic acid results.

Because of the intermittent nature of MS, a single specimen can give us only a cross-sectional view of a surmisedly unstable situation. In view of the possibility that deviations in level of serum and CSF constituents may be detectable only during acute episodes, the observed incidence of elevated values is as high as may be expected and gains considerable significance. High vitamin values in CSF may be involved in the etiology of MS, or may act merely as indicators of changes in permeability of the blood-brain barrier. That this permeability is subject to variations was observed in a collateral study, where very high PGA and B_{12}-levels in CSF of neurological patients dropped abruptly within 5 min. during electric shock therapy, indicating a temporary breakdown of the blood-brain barrier. These observations support the concept that

vitamin B_{12} is involved in various pathological cerebral manifestations(8), which are ultimately based on enzymatic disturbances. Decreases and increases(9) of various hydrolases in degenerating nervous tissue have been reviewed by Cavanagh and Thompson (3). Some of these enzymes (*e.g.* the glycosidase synthesizing nucleosides) as well as transmethylase are interrelated with B_{12} and PGA metabolism.

Summary. The folic acid and Vit. B_{12} content of CSF and B_{12} in serum was studied in more than 300 neurological cases. The PGA levels in CSF in a number of cases were elevated; high values were scattered among a variety of neurological conditions, but were high in four-fifths of cases with MS. The titre of B_{12} in CSF and serum of MS patients was elevated in one-third and reached the upper limit of the normal range in another third of the cases. We assume that increased levels of these vitamins in CSF are not merely an indication of increased permeability of the blood-brain barrier, but of etiological significance for MS.

1. Sobotka, H., *Clin. Chem.,* 1958, v4, 93.
2. Johnson, B. C., *Am. J. Clin. Nutr.,* 1958, v6, 34.
3. Cavanagh, J. B., Thompson, R. H. S., *Brit. Med. Bull.,* 1954, v10, 47.
4. Hutner, S. H., Cury, A., Baker, H., *Anal. Chem.,* 1958, v30, 849.
5. Baker, H., Hutner, S. H., Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 210.
6. Hutner, S. H., Bach, M. K., Ross, G. I. M., *J. Protozool.,* 1956, v3, 101.
7. Baker, H., Sobotka, H., Pasher, I., Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 636.
8. Holmes, J. M., *British Med. J.,* 1956, v2, 1394.
9. Hollinger, D. M., Logan, J. E., Mannell, A. W., Rossiter, R. J., *Nature,* 1952, v169, 670.

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Effect of Total Adrenalectomy on Benzol-Induced Leukopenia in Rabbits.* (24098)

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Selling(3) was one of the first investigators to show that following acute benzol poisoning in rabbits there is a prompt fall in circulating white blood count following which, if sepsis and death do not intervene, there is a gradual rise to normal levels after 2-3 weeks. He also noted that, while there was considerable variation in response of various animals to toxic action of the drug, the general response was a fall to low levels following which there was a preliminary rise, followed by a fall, and then again by a secondary rise which gradually reached normal levels. He believed that this phenomenon was probably dependent upon 2 factors: (1) rapidity of recovery of regenerative organs, and (2) amount of toxic material still present in circulating blood.

Weiskotten(4) also noted uniform but complex response of leukopenia in rabbits to acute benzol poisoning and called the phenomenon a "Diphasic Leucopenia." He named the primary fall and rise the "Protophase," the secondary fall and rise the "Deuterophase." He found that the response was not due to antigen-antibody reaction, nor was it due to amount of toxic material present in the circulating blood or delayed absorption of the substance. He also found that in the presence of a pre-existing quiescent infection there was a "lighting up" of the infectious process following injection of benzol and the leucocyte curve behaved in an irregular fashion. The leukocyte count characteristically rose to abnormally high levels despite acute benzol poisoning. Weiskotten and his co-workers were unable to determine the cause of the characteristic diphasic curve. Harkins(2) investigated the effect of nucleotide K96 upon the benzol induced leukopenia and found no significant alterations of the curve. Since a satisfactory explanation for the "diphasic" response has

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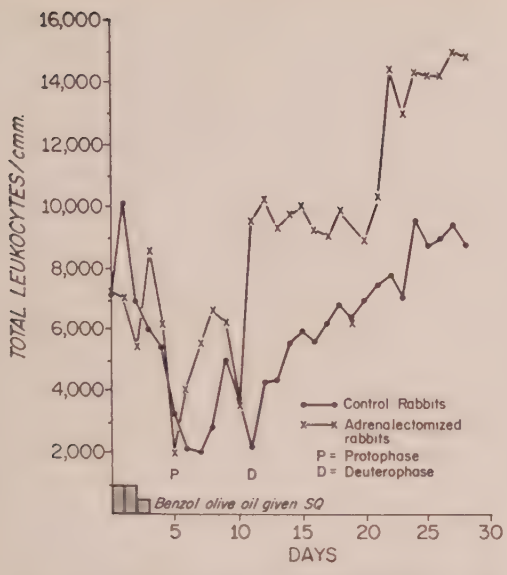

FIG. 1. White blood count changes following subcutaneous administration of benzol-olive oil mixture. (Representative pair of rabbits are from each of the 2 series—control and adrenalectomized.)

not been found an attempt to evaluate the role of the adrenal glands in this phenomenon was devised in the following experiment.

Method. Bilateral total adrenalectomy was successfully performed on 3 male rabbits weighing from 3.5-4.5 kg. Attempts were made to perform a bilateral, one stage adrenalectomy after the method described by Zak, Good and Good(5), but we had a 100% mortality. After losing a large number of animals (approximately 20 rabbits) in the postoperative period, we followed the method described by Criep, Mayer, and Lozano Menchaca(1) except that we gave an initial hypnotic dose of pentobarbital (30 mg/kg) intraperitoneally and did not give intravenous fluids. Postoperatively the animals were given 25 mg cortisone acetate and 5 mg DOCA intramuscularly for one dose. Saline was substituted for water in the diet. With these exceptions the animals were treated and fed in the same manner as the control animals.

The 3 adrenalectomized animals and the control animals were given subcutaneous injections of 1.3 cc/kg of a solution of equal parts chemically pure benzene and olive oil. A full dose was given on each of the first 2 days and only half a dose on the third day. Daily white blood counts were done on all of the rabbits for 1 month.

Results. The comparative results in a representative pair of rabbits are shown in Fig. 1. The characteristic diphasic curve is similar in the experimental and the control animal. The results in the other 2 pairs of rabbits were similar. This would appear to indicate that the adrenal glands have no significant role in producing either the protophase or deuterophase portions of the phenomenon.

Summary and conclusions. No significant relationship has been found between the diphasic response in the benzol-induced leukopenia in rabbits and the presence or absence of adrenal glands.

1. Criep, L. H., Mayer, L. D., Lozano Menchaca, O. E., *J. Allergy,* 1951, v22, 314.

2. Harkins, H. N., *J.A.M.A.,* 1932, v99, 1132.

3. Selling, L., *Johns Hopkins Hosp. Reps.,* 1916, v17, 83.

4. Weiskotten, H. G., *J. M. Research,* 1915-1919, 33; *Am. J. Path.,* 1930, v6, 183.

5. Zak, S. J., Good, R. H., Good, R. A., *Nature,* 1957, v179, 100.

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Collection and Liquefaction of Guinea Pig Semen.* (24099)

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Investigations of the physiology of reproduction in the male guinea pig and rat have been hampered by lack of methods for semen collection and for "vaginal plug" liquefaction. Consequently, work with these animals has been limited to study of histological and biochemical changes in testes and accessory glands. The 2-fold purpose of this study was to develop a routine method for collection of semen specimens at regular intervals from the guinea pig and to find a simple procedure for plug liquefaction, so that changes in semen characteristics might be used in investigations of this type. Use of electroejaculation for collection of bull and ram semen is now common practice and has been adequately reviewed(1). Dalziel and Phillips(2) developed a technic for electroejaculation of chinchillas and guinea pigs but did not report on the material collected. This paper describes the apparatus and methods used for regular collection of guinea pig semen by electroejaculation and use of proteolytic enzymes in liquefaction of resultant semen coagulum.

Methods. Eighteen mature albino guinea pigs were used to collect 110 semen specimens by electroejaculation during 4 month period. The hair was clipped from lumbar region and electrode jelly applied to clipped area. The guinea pig was strapped on its back to animal board through which there was inserted a round copper disk (diameter 1.75 cm) used as lumbar electrode. The anal electrode was a smooth brass tube (diameter 0.4 cm; length 8.0 cm) lubricated with K-Y jelly and inserted 4-6 cm into the rectum. A simple and inexpensive electronic setup was designed to deliver an intermittent square wave, 25 volt rms. at 1000 cycles, with automatic 3 second "on period" and 12 second "off period" (Fig. 1). The mean number of shocks required for ejaculation was 5 (range 3-14 shocks). The semen specimen was collected in a recalibrated 5 ml graduate in 1 ml of phosphate buffer after White(3), containing appropriate concentration of the enzyme under study; semen volume was read to nearest 0.1 ml. Motilities were estimated under low power of microscope in a slide warmer at 39°C. There was no apparent ill effect of biweekly collection, although as many as 8 specimens were collected from each of 5 animals during the experimental period. While muscle contraction and squealing were noted in the 3 second "on period" during the early part of the study, the effect of conditioning became apparent towards the latter part of the study when there were little or no visible or audible signs of distress during the "on period."

Results. Preliminary trials indicated that semen coagulated almost instantaneously upon ejaculation and in some cases it was ejacu-

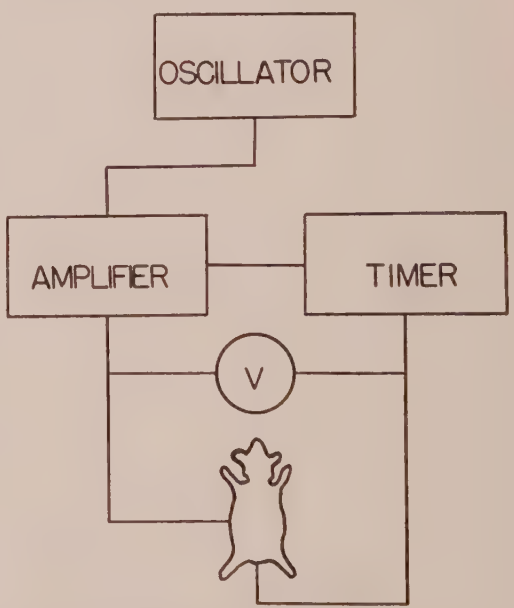

FIG. 1. The oscillator produces a 2.5 volt, 1000 cycle, square wave from the 110 volt, 60 cycle AC line and output at the amplifier is set at 25 volts. The cam operated timer is designed to deliver a 3 sec. "on period" and a 12 sec. "off period."

* This work was supported in part by grant from U.S.P.H.S.

lated in plug form, which indicated that it had coagulated in the urethra. This semen coagulum did not spontaneously liquefy even after 6 hours incubation at 39°C. When the coagulum was collected in Ringer's solution or in phosphate buffer, there was no indication of liquefaction at any time during 6 hours at 39°C.

A series of commercially available enzymes were then tried for their liquefying activity in this system.† Since Bunge and Sherman (4) had shown that alpha-amylase would liquefy coagulated human semen specimens, it was tried in Ringer's solution and in phosphate buffer, at concentrations ranging up to 10%. Partial but incomplete liquefaction was noted in 8 specimens with some cells free of the coagulum and feebly motile. Varidase‡ dissolved in phosphate (17,000 units Streptokinase and 10,000 units Streptodornase/ml) was tried with 9 specimens and in every case there was complete liquefaction of the coagulum and vigorous sperm motility. Within minutes, however, recoagulation was noted on the slide and in the collection graduate and, in 5 to 10 minutes (at 39°C), all specimens were completely recoagulated. This phenomenon of recoagulation, as observed under the microscope at 39°C, was made up of several stages; 1. completely liquefied semen was free of coagulum and the cells moved freely and vigorously; 2. small centers of coagulation appeared uniformly throughout the field and the forward motion of the sperm was markedly decreased, although their tails continued beating rapidly; 3. centers of coagulation enlarged to occupy most of the field and there was no forward motion although many tails continued to beat feebly; 4. the coagulum occupied the entire field enmeshing the sperm and no tail motion could be observed. When an additional ml of Varidase was added and stirred into the coagulum, reliquefaction took place and the sperm regained vigorous motility. This was followed by complete recoagulation within 5 to 10 minutes on the pattern described.

Use of bromelain, papain, cathepsin, or trypsin in concentrations ranging to 0.1% in phosphate yielded variable results, with partial liquefaction of the coagulum in some specimens and no liquefaction in others.

Chymotrypsin was tried at concentrations ranging from 0.001 - 0.1% in phosphate. At the 0.1% concentration it consistently produced liquefaction and the specimens did not recoagulate during 6 hours of incubation at 39°C. At lower concentrations of chymotrypsin, liquefaction was incomplete and, in a number of cases, recoagulation took place. Of the 78 specimens collected in 0.1% chymotrypsin-phosphate, 69 liquefied rapidly and contained motile cells (mean motility = 51%; range = 10-90%), 4 did not liquefy, 4 liquefied but showed no sperm motility, and 1 specimen was sperm free. The motility of these cells was characterized by curvilinear swimming patterns, irregular forward motility, and "head to head" rouleaux. Mean ejaculate volume was 0.5 ml (range 0.1-1.2 ml).

During the 4 month study, a marked trend was noted for the ejaculated semen to remain liquid and fail to clot, *i.e.* those pigs that had been electroejaculated several times, at biweekly intervals, began to deliver specimens that did not coagulate at ejaculation or even after several hours of incubation. The liquid semen specimens were either completely coagulum free or else had only small, scattered islands of coagulum and the sperm motility was, in all cases, good. The possibility that the failure of the semen to coagulate was due to damage to the accessory glands by repeated electroejaculation was investigated by histological methods. Two "electroejaculate" pigs and 2 "control" pigs (breeders that had never been electroejaculated) were sacrificed and tissue from the coagulating gland, dorsal and lateral prostate, seminal vesicle, testis, and rectum was fixed in Bouin's, sectioned at 4 μ, and stained with hematoxylin and eosin. There was no evidence of gross morphological or histological differences between the "electroejaculate" and the "control" pigs.

† Trypsin, chymotrypsin, alpha-amylase (Nutritional Biochemicals); trypsin, chymotrypsin, alpha-amylase (Bios Labs); bromelain, papain, cathepsin, pepsin (Mann Research Labs).

‡ We are indebted to Dr. J. M. Ruegsegger, American Cyanamid, N. Y. City, for the Varidase.

It might be suggested that the ejaculation of liquid semen after repeated biweekly electroejaculation is the result of some physiological change affecting either vesiculase production by the coagulating gland or else some other factor involved in the clotting mechanism. This might be due either 1) to frequency of ejaculation or 2) to electroejaculation of larger volumes of semen than might be delivered under the normal conditions of coitus, with the result that the ability of the accessory gland system to regenerate some essential part of the clotting mechanism is exceeded. A further investigation of this problem will use the addition of a purified vesiculase to these liquid specimens to determine whether failure to coagulate is due to a lack of vesiculase.

Summary. A method and an apparatus for routine electroejaculation of guinea pigs were developed, the semen coagulum or "vaginal plug" examined, and the coagulation process described. A series of proteolytic enzymes were tried for their liquefying activity and 0.1% chymotrypsin was found to result in consistent liquefaction and good sperm motility. Mean ejaculate volume was 0.5 ml and mean motility at 39°C was 51%. There was no evidence of gross morphological or histological changes in the reproductive system of guinea pigs electroejaculated at biweekly intervals.

I am indebted to Mr. Lloyd Schoenbach for the design and construction of the electroejaculator and to Mrs. Olga Radimska for histological preparations.

1. Emmens, C. W., Blackshaw, A. W., *Physiol. Rev.*, 1956, v36, 277.

2. Dalziel, C. F., Phillips, C. L., *Am. J. Vet. Res.*, 1948, v9, 225.

3. White, I. G., *Austral. J. Biol. Sci.*, 1954, v7, 379.

4. Bunge, R. G., Sherman, J. K., *Fertility and Sterility*, 1954, v5, 353.

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Apparatus for Continuous Intravenous Infusion in Dogs. (24100)

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Several types of apparatus for intravenous infusion of fluids in dogs have been developed, each designed to meet particular needs of different investigations(1,2,3). The main feature of the apparatus described here is a standard assembly consisting of a reservoir bottle, drip device and connecting tubes leading to indwelling intravenous catheter, commonly employed in clinical hospital practice. The polyethylene catheter is introduced into an external jugular vein as described by Zimmermann(4). The bottle is suspended under a swivel, with a flexible support for the connecting tubing arranged so that the dog has almost complete freedom of movement in a spacious cage while fluids are infused during varying periods of time.

Apparatus, Fig. 1: *1.* Pulley roller on ¾ inch round rod, held 4 feet above top of cage, permits backward and forward movements of dog. *2.* Swivel hook hanging below pulley, turns freely through a complete circle. *3.* Square ⅜ inch steel rod, with hook at top, suspended from swivel. *4.* Light coil spring, fastened at its top to the rod by adjustable clamp and at its lower end to a bracket (*5*), which slides up and down on rod. Attachments of spring are adjusted to give tension strong enough to hold the bracket, tubing and harness at a level comfortable for the dog. *5.* Sliding bracket with 2 square holes fitting the steel rod loosely. Excursion of the bracket

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downward is limited by adjustable cotter pin placed in one of several holes at 2-inch intervals in rod. *6*. A reservoir bottle, suspended from an arm at top of square rod, delivers fluid through a standard infusion set with drip device and adjustable screw clamp on tubing for control of flow. That illustrated is the Mead Johnson Co. disposable unit. To permit finer regulation of flow a standard tunnel clamp can be substituted for the simple screw clamp shown. *7*. Flexible metal tubing (BX electric conduit) through which passes the connecting rubber or plastic tubing. This metal conduit is secured to the sliding bracket (*5*) and to a universal joint (*8*), above dog's harness. *8*. Universal joint which permits 4-way flexing movements, connected by a detachable bayonet lock to a post (*8a*) mounted on metal plate (*9*) on dog's harness. *9*. Metal plate on which is mounted the post (*8a*) with bayonet lock, and another post (*8b*) with a clamp for holding the hilt of a needle on which the plastic catheter (*10*) is fitted. *10*. Polyethylene catheter leads from the needle into an external jugular vein, with a generous coil to allow the dog's head ample freedom. *11*. Harness, with adjustable leather straps. The polyethylene catheter is sterilized by filling and soaking in a solution of 1:1000 Zephyran solution a few hours. It is then rinsed with salt solution and left filled with heparin-saline solution, with attached needle and plug.

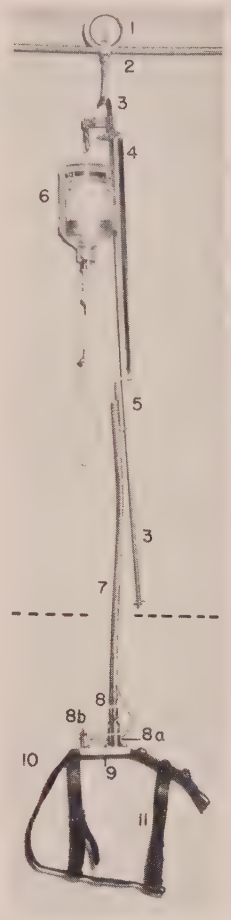

FIG. 1. Assembly of dog's harness and supports for reservoir bottle and intrav. drip apparatus.

Procedure. Clip closely the hair around dog's neck from shoulders to back of head. With the dog tied, either back down or on one side, place 1-inch band of adhesive tape partly around neck just above a point selected for entering the external jugular vein. At that point inject novocaine intradermally and allow time for the anesthetic effect. With a scalpel point make a small incision through the skin over the vein. With syringe attached to 14 or 15-gauge needle, insert needle through incision into the jugular vein. Withdraw some blood to prove a good entry. Remove syringe and quickly insert the polyethylene catheter tubing through the needle and 8 or 10 inches into the jugular vein. Inject a few cc of heparin-saline solution, then plug tubing with the wire. Remove the 15-gauge needle from jugular vein while pressing a finger on skin over the needle tip in the vein to hold the catheter in place. Wipe the catheter with saline soaked gauze to remove blood. For protection, place over the catheter a rubber or plastic covering tube, just long enough to reach the back of the neck behind the occiput, leaving 12 or 15 inches of the catheter tubing free. With short strips of adhesive tape, anchor this covering tubing to the skin and to the adhesive tape that was first placed around the neck. Make a firm protective collar by wrapping 3-inch gauze bandage around the neck several times. Fasten the gauze securely in place with 2-inch wide bands of superimposed adhesive tape. Place the harness on the dog and tighten straps to fit

snugly, with the dog standing up. Remove the wire plug from the tubing, insert a 21-gauge needle and again inject some heparin-saline solution. Insert a plug in the hilt of the needle and clamp the hilt on the supporting post (*8b* in Fig. 1).

Place dog in the cage, above which the assembled perfusion apparatus has already been suspended. Connect the metal conduit tubing to the supporting post (*8a*) by means of the bayonet lock. Remove the plug from the needle and insert the adapter tip of the tubing of the drip apparatus. Open the adjustable screw clamp to allow drops of fluid to flow at a chosen rate.

Discussion. The design of this apparatus is similar to one developed in this laboratory several years ago and used successfully in studies on effects of ammonium chloride acidosis on insulin action(5) and on glucose tolerance(6). In the earlier version a bicycle chain was used to support the tubing between reservoir and connection to the indwelling intravenous catheter, to allow up and down movements of the dog while protecting against twisting of the tubing, as was first employed by Jacobs(1). The flexible metal conduit as now used serves the same purpose but is simpler and permits easier assembly of the tubing than did the bicycle chain.

Summary. This report describes a simple apparatus for continuous intravenous infusion of fluids in dogs, employing a standard assembly of reservoir bottle and flexible connecting tubing that leads to the dog's harness and a plastic catheter dwelling in an external jugular vein. Suspension of the apparatus by a swivel above the dog's cage permits the dog almost complete freedom of movement during long periods of continuous infusions.

1. Jacobs, H. R. D., *J. Lab. and Clin. Med.,* 1931, v16, 901.

2. Stengel, A., Jr., Vars, H. M., *ibid.,* 1939, v24, 525.

3. Rhode, C. M., Parkins, W., Tourtellotte, D., Vars, H. M., *Am. J. Physiol.,* 1949, v159, 409.

4. Zimmermann, B., *Science,* 1945, v101, 567.

5. Mackler, B., Lichtenstein, H., Guest, G. M., *Am. J. Physiol.,* 1951, v166, 191.

6. ———, *ibid.,* 1952, v168, 126.

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Some Effects of Aureomycin and Penicillin on Thiamine and Riboflavin Metabolism in Growing Rats.* (24101)

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Investigators have found that certain antibiotics stimulate growth in young animals of several species but the mode of action has not been fully elucidated. It has been shown that the resulting growth response depends on the antibiotic used, animal species, composition of diet, and on sanitary condition of the environment. In general, antibiotics are less effective in stimulating growth when fed with nutritionally complete diets to animals maintained under sanitary conditions. These observations suggest a relationship between growth-promoting effect of antibiotic and intestinal flora of the animal. Lih and Baumann (1) reported vitamin-sparing action occurring in rats subsisting on antibiotic-containing diets sub-optimum in certain water-soluble vitamins. They reported penicillin to be more effective in stimulating additional growth in rats maintained on thiamine or riboflavin-deficient diets than are other antibiotics whereas in pantothenic acid deficiencies penicillin was least effective. Sauberlich(2) reported the thiamine-sparing action of aureomycin or penicillin to be greater in rats receiving dextrin-containing diets than in rats receiving sucrose-containing diets. He also reported the effect of these antibiotics to be greater when

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used in connection with thiamine deficiencies than with riboflavin or pyridoxine deficiencies. Johnson *et al.*(3) suggested the function of penicillin in sparing dietary thiamine to be through increased synthesis of the vitamin. Guggenheim *et al.*(4) investigated the effect of parenterally and orally administered antibiotics. According to these authors, antibiotics administered subcutaneously exhibited no significant growth-stimulating in animals receiving sub-optimal levels of thiamine, riboflavin, or pantothenic acid. When given orally to rats receiving sub-optimal levels of thiamine, penicillin and terramycin stimulated additional growth whereas aureomycin and streptomycin failed to do so. However, when given orally to riboflavin and pantothenic acid-deficient rats, penicillin, aureomycin, streptomycin, and terramycin were effective. Oral administration of antibiotics also affected excretion of vitamins. Linkswiler *et al.*(5) reported that orally administered aureomycin stimulated growth in rats receiving diets deficient in the B_6 vitamins, whereas Waisman *et al.*(6) found that this antibiotic overcame citrovorum factor deficiencies induced by feeding aminopterin. Much experimental work with antibiotics concerns growth of test animal when antibiotics are administered under controlled conditions. Except the efforts of Guggenheim *et al.*(4), little seems to have been done toward establishing a relationship between vitamin intake of test animal and that stored in tissues or that eliminated in excreta in presence or absence of ingested antibiotics. The present report contains some of the results obtained with rats relative to thiamine and riboflavin absorption and retention in the presence and absence of dietary aureomycin and penicillin.

Methods. Rats 20 to 28 days of age and weighing from 36 to 53 g were used. All rats except normal controls were partially depleted of body-store of thiamine, riboflavin, or thiamine and riboflavin, before being arranged on the basis of sex, weight, age, and genetic background into comparable experimental groups. The basal diet consisted of: vitamin-free casein 17%, hydrogenated cotton-seed oil 5%, salt mixture (U.S.P.) 4%, fiber (CellU-flour) 2%, cod liver oil 1%, vitamin pre-mix 1%, and sucrose 70%. The vitamin pre-mix contained 100 g : alpha tocopherol 2.5 mg, pyridoxine hydrochloride 0.25 mg, p-amino benzoic acid 20 mg, niacin 2 mg, choline chloride 100 mg, inositol 20 mg, calcium pantothenate 2 mg, folic acid 0.4 mg, Vit. K 0.2 mg, Vit. B_{12} 0.004 mg, and biotin 0.025 mg, dispersed in finely-pulverized casein. The antibiotics were incorporated into the basal diet at the rate of 100 ppm. When 2 antibiotics were incorporated into the same diet, 100 ppm of each were used. The 2 antibiotics employed were: penicillin (Procaine penicillin—Merck and Co.) and aureomycin (Lederle Laboratories). The supplementary vitamins (thiamine and riboflavin) were fed orally as freshly-prepared acidulated aqueous solutions. Experimental diets were prepared at biweekly intervals and stored under refrigeration until fed *ad libitum*. Rats were maintained in individual all-metal cages having raised-screen floors except during periods of excreta collecting. During the latter periods, they were maintained in individual metabolism cages permitting quantitative collection of urine and feces. Rats were weighed daily and food-consumption data were recorded weekly. Excreta from individual animals were collected daily, weighed or measured and preserved by means of 0.1 N hydrochloric acid and refrigeration. Four to 8 animals were used in each dietary treatment. At end of feeding periods, animals were sacrificed and liver and ceca (including contents and 10 mm of intestines on portal and ventral side) were dissected out, pressed between filter papers, weighed, macerated in presence of 0.1 N hydrochloric acid, and transferred to volumetric flasks. Feces were similarly treated. Urine, feces and body tissues were subjected to hydrolysis, made to volume with dilute acid, and aliquots of solutions were adsorbed on appropriate columns, eluted, and eluates assayed for thiamine and riboflavin by fluorometric procedures using Coleman Model 12 fluorometer.

Results. Data are reported (Table I) relative to food intake, body-weight increase, and amounts of thiamine and riboflavin recovered from liver, ceca, urine, and feces of rats following ingestion of known amounts of the 2

TABLE I. Some Effects of Penicillin and Aureomycin on Thiamine (B_1) and Riboflavin (B_2) Metabolism in the Growing Rat.

Animal group No.	I		II		III		IV		V		VI		VII		VIII	
Sub-group	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Previously depleted of	$B_1 + B_2$				B_1				B_2				B_1		B_2	
Daily thiamine intake (μg)*	2	2	1	1	2	2	12.5	12.5	12.5	12.5	12.5	12.5	2	2	12.5	12.5
" riboflavin " " *	0	0	4	4	25	25	25	25	1	1	25	25	25	25	2	2
Type of antibiotic fed†	P	None	P	None	P	None	P	None	P	None	P + A	None	A	None	A	None
Total food consumed (g)‡	89	69	81	49	95	81	151	194	187	166	215	208	120	121	147	127
" wt incr. (g)‡	31	11	28	7	31	18	72	100	45	45	88	82	18	17	22	12
Food consumed/wt incr.‡	2.9	6.3	2.9	7	3.1	6.2	2.1	1.9	4.2	3.8	2.4	2.5	6.7	7.1	6.7	10.6
Thiamine conc. (μg/g sample)																
Liver	2.4	1.6	1.3	0.7	1.7	1.1	3.4	2.5	3.2	3.6	3.1	3.4	1.1	1.2	3.9	3.6
Ceca	.2	.7	.2	.8	1.4	.7	.5	.9	.6	.6	.4	.8	.4	.4	.9	1.8
Feces	1.4	4.8	2.5	4.2	1.6	3.4	1.8	2.2	2.5	3.6	1.3	3.5	.8	1	2.1	3.8
Urine	.2	.2	.3	.4	2.9	1.2	1.7	.6	.2	.8	1.3	.8	.2	.1	.4	.3
Total thiamine (μg)‡																
Intake	40	40	20	20	40	40	250	250	250	250	250	250	40	40	250	250
Feces + urine	5.9	13.9	7.5	10.6	7.5	13.3	10.6	17.8	17.2	24.2	12.7	23.5	4	4	12.3	14.2
Liver + ceca	7.4	4.9	4.8	2	7.7	6.6	19.9	17.7	20.2	22.5	21.7	20.6	6.1	7.3	20.2	20.9
Riboflavin conc. (μg/g)																
Liver	8	7.6	8.8	10.8	13.8	26.6	16.9	16.3	11.8	11.2	15.4	14.6	20.8	18.5	9.8	8.8
Ceca	2	3	1.9	6.2	4.4	5.5	4.8	5.6	2.3	2.5	2.5	2.8	2	1.7	2.6	3.6
Feces	6.6	11.6	5.4	12.4					6.4	7.1	10	11.6	15.3	22.8	5.9	7.5
Urine	7.8	5.	4.3	5.6					2.6	3.2	10.3	7.3	26	20.9	6.7	8.4
Total riboflavin (μg)‡																
Intake	0	0	80	80	500	500	500	500	20	20	500	500	500	500	40	40
Feces + urine	46	51	29	44					59	61	113	114	121	113	56	50
Liver + ceca	28	25	36	26					69	74	112	139	93	118	51	47

* Supplements fed daily 20 days. † A = Aureomycin; P = Penicillin. ‡ Avg value for 21-day test period.

vitamins in presence and absence of the 2 antibiotics. The data reported are average values for the several rats constituting the respective experimental groups.

Discussion. In 6 out of 8 experiments, rats receiving diets supplemented with antibiotics consumed more food and grew at a more rapid rate (increase in body-weight) than did those receiving unsupplemented diets. Efficiency of food utilization likewise was highest among rats receiving antibiotic-supplemented diets in 6 experiments out of 8. In the other 2 experiments, where thiamine intake was high, food efficiency was slightly higher on the control diets. In all experiments antibiotics decreased the total amount of ingested thiamine recovered from the liver, ceca, feces, and urine. However, in no instance was the total amount of thiamine recovered equal to that ingested. Antibiotic supplementation increased thiamine content of the livers in 5 of 8 experiments and decreased the thiamine content of the ceca in 5 experiments. Antibiotics decreased the thiamine content of feces in all experiments and increased the thiamine content of the urine in 5 of 8 experiments. In 5 of 6 experiments, antibiotics decreased the total amount of ingested riboflavin accounted for in liver, ceca, urine, and feces of rats. When low dosages of riboflavin were given (0, 1, and 2 μg daily), the total amount of vitamin accounted for in the 4 sources was greater than the amount ingested. It is not clear whether this indicates synthesis of vitamin or whether the difference may have been due to residual riboflavin unremoved by previous depletion. If riboflavin synthesis took place, the synthesis was not influenced by presence or absence of the antibiotics. In 6 of 8 experiments, concentration of hepatic riboflavin was highest in rats having received the antibiotic-supplements, the difference being greatest where high intakes of the vitamin were involved. Concentration of riboflavin in ceca and the feces was generally lowest in rats that received the antibiotic supplements. However, in 3 experiments urinary riboflavin was highest for these rats.

Summary. 1. The data demonstrate that both penicillin and aureomycin supplementation increased food intakes of rats subsisting on diets deficient in either or both thiamine and riboflavin, but neither antibiotic influenced food consumption when adequate thiamine and adequate riboflavin was administered. In general, growth of test animals paralleled food consumption, with indications that antibiotic supplementation improved food utilization, particularly when thiamine intake was sub-optimum. 2. The data further indicate that the amount of thiamine accounted for was increased by feeding rats diets containing either penicillin or aureomycin, or a combination of the 2 antibiotics. However, the data show that thiamine absorption was improved in the presence of antibiotics, particularly, penicillin, as indicated by lower thiamine concentration in ceca and feces, and by higher thiamine concentration in liver and urine. 3. While the data do not exclude the possibility of riboflavin synthesis in the animal body, they do indicate that if synthesis occurred it was not influenced by the presence or absence of the antibiotics under investigation. There was evidence of increased riboflavin absorption in the presence of antibiotics as indicated by lower concentrations of vitamin in ceca and feces and a higher concentration in liver.

1. Lih, H., Baumann, C. A., *J. Nutrition,* 1951, v45, 143.

2. Sauberlich, H. E., *ibid.,* 1952, v46, 99.

3. Johnson, B. C., Schendel, H. E., Hartsook, E. W., Batchlor, B., Promislow, C., Cohn, E. M., *Am. Chem. Soc. Abst.,* 1953, 26A.

4. Guggenheim, K., Halevy, S., Hartmann, I., Zamier, R., *J. Nutrition,* 1953, v50, 245.

5. Linkswiler, H., Baumann, C. A., Snell, E. E., *ibid.,* 1951, v43, 565.

6. Waisman, H. A., Cravioto-M, J., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 525.

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Liquid Scintillation Counting of C^{14} and H^3 in Plasma and Serum. (24102)

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Liquid scintillation technics for counting low energy β-emitting isotopes have proven to be sensitive, simple and rapid. A basic requirement for such counting is to dissolve the radioactive material in a phosphor-containing organic phase, usually toluene. Several general solutions to the problem of counting substances of low solubility in toluene include (a) the incorporation of ethanol, for counting of aqueous phases and (b) utilizing the complexing ability of a quaternary ammonium base, Hyamine for counting CO_2(1) amino acids and proteins(2). In this report, a combination of these technics has been employed to determine the total serum content of C^{14} and H^3 with accuracy and rapidity.

Methods. Apparatus and materials. Solutions were counted in the Packard Tri-Carb Model 314-DC Liquid Scintillation Spectrometer with counting chamber set at –8°C. Carbon^{14} was counted at photomultiplier voltage Tap 6 (1060 volts) and Tritium at Tap 10 (1390 volts). The pulse-height discriminators were set at 10-100 volts. Efficiencies with C^{14} and H^3 using 5.0 ml of toluene-phosphor were 68% and 21% respectively. Hyamine was prepared by the method of Eisenberg(3). Commercial Hyamine, 10X, Rohm and Haas, Philadelphia [p-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium chloride] was recrystallized 4 times from 4 volumes of toluene, dried, and converted to free base by shaking the chloride (96 g) with silver oxide (25.5 g) and water (1.8 ml) in methanol (100 ml) for exactly 10 minutes. After centrifugation, the supernatant was exposed to sunlight for several days and recentrifuged. The preparation was 0.97 M. *Recommended procedure.* To one ml of Hyamine in a counting vial (5-dram, Wheaton Glass Co., Millville, N. J.) is added 0.1 ml plasma or serum. The vial is then rotated to dissolve the plasma proteins. (Addition of Hyamine to protein may result in a protein coagulation which requires heat to dissolve.) To the vial contents is then added 0.5 ml ethanol and 5 ml toluene containing 0.6% diphenyloxazole (DPO) and 0.02% 1,4-di [2-(5-phenyloxazolyl)] benzene (POPOP). The vial is gently rotated to produce a homogeneous solution, cooled to the temperature of the counting chamber (–8°C) and counted at the appropriate voltage settings for C^{14} or H^3. An internal standard is then added and the observed count corrected for the overall quenching caused by the protein, water, ethanol and Hyamine content.

Results. Measurement of total activity in plasma or serum without extensive sample preparation demanded a method for rapid solution of the sample in a scintillating medium. Hyamine was a suitable complexing agent for the plasma proteins, but the technic has certain limitations.

The plasma protein concentration may vary from one sample to another, giving rise to slight variations in the degree of quenching. Superimposed on this variable is the quenching which may be caused by hemolysis. In Table I is shown the C^{14} count of 10 aliquots of 0.1 ml serum from one sample. The standard deviation was $\pm$ 1.10%, only slightly greater than the standard error of counting 14,000 counts which is $\pm$ 0.85%. However, in counting 10 different samples of serum containing equivalent activities, the standard deviation was $\pm$ 3.6%, considerably greater than the counting error of $\pm$ 0.5% in these counts (>40,000 counts). It was noted that hemolyzed serums counted 2-5% less than unhemolyzed serums.

Adequate correction for quenching is effected by the counting of an internal standard. Table II shows the counting of 0.1-0.4 ml volumes of serum containing C^{14} or H^3. Each serum sample was easily dissolved by 1.0 ml of Hyamine, but the larger samples required more ethanol to form a single phase. In these samples, the greater quenching resulted in an observed cpm/0.4 ml only 50% greater

TABLE I. C^{14} Counting of Serum.

Sample	No. samples	Avg observed counts/min.	Stand. dev. (counts/min.)	Stand. dev. (%)	S.E. counting (%)
.1 ml replicates	10	7276	80.2	1.10	.85
.1 " different	10	6347	229	3.6	.5

TABLE II. Liquid Scintillation Counting of C^{14} and H^3 in Serum.

Isotope*	ml serum	ml ethanol	C.P.M., −bkgd.†	Increment owing to addition of internal std.	Overall efficiency (%)	C.P.M./ml serum
C^{14}	none	none		10,630	68	
	.1	.5	4,367	5,326	34.1	87,200
	.2	1.5	5,318	3,296	21.1	85,900
	.3	2.0	6,362	2,494	16.0	90,200
	.4	2.5	6,473	1,918	12.3	89,800
H^3	none	none		35,940	21	
	.1	.5	6,442	6,276	3.67	369,000
	.2	1.5	8,200	4,116	2.40	358,000
	.3	2.0	9,663	3,316	1.94	349,000
	.4	2.5	10,181	2,403	1.41	380,000

* C^{14} counted at Tap 6 (1060 volts), 10-100 volt window; H^3 counted at Tap 10 (1390 volts), 10-100 volt window.

† Bkgd. C^{14} 18.0 cpm.; bkgd. H^3 47.2 cpm.

than the observed cpm/0.1 ml serum. This points to the definite limitation of sample size. Specific activities as corrected by internal standard (cpm/ml serum) were nevertheless constant as seen in Table II.

For 0.1 ml volumes of serum, the 34% efficiency for C^{14} and 3.7% efficiency for H^3 compares favorably with other methods of measuring total activity. Background counts in the scintillation counter were 18.0 cpm. and 47.2 cpm for C^{14} and H^3 respectively.

Previously described technics for determination of total C^{14} radioactivity are either accurate but of low efficiency (counting of infinitely thick liquid samples with thin-window flow counters)(4) or sacrifice accuracy for simplicity (plating methods). The latter technics require self absorption corrections which are often of poor reproducibility and electrostatic effects may seriously disturb counting of dried plasma samples with a windowless counter. We are not aware of any other simple technic for counting total H^3 activity in plasma.

Summary. A liquid scintillation technic for counting total plasma or serum activity of C^{14} and H^3 is described. The serum is complexed by the quaternary ammonium base, Hyamine and dissolved in phosphor-containing toluene with the addition of ethanol. Correction for quenching is effected by recounting an added internal standard after determination of the observed count. The method was shown to be rapid and accurate for various volumes of serum.

1. Passmann, J. M., Radin, N. S., Cooper, J. A. D., *Anal. Chem.*, 1956, v28, 484.

2. Vaughan, M., Steinberg, D., Logan, J., *Science*, 1957, v126, 446.

3. Eisenberg, F., Northwestern Univ. Conf. Liquid Scintillation Counting, 1958. Pergamon Press, N. Y. City.

4. Cotlove, E., *Fed. Proc.*, 1955, v14, 32.

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Elevation of Peripheral Blood Ammonia Following Muscular Exercise. (24103)

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Hyperpnea observed during and immediately following muscular exercise has been ascribed to changes in blood pH, PCO_2, anoxia, and reflexes from exercising muscles or great veins. These alterations do not appear to account completely for the intensity of hyperpnea(1). It has been suggested, therefore, that there is still an unrecognized respiratory stimulant contributing to increase in ventilation during and following muscular exercise. The possible role of ammonia in this respect is worthy of consideration. Respiratory alkalosis in patients with elevated blood ammonia due to hepatic coma has been well documented. Infusion of neutral ammonium salts into normal dogs also produces respiratory alkalosis(2). It is known, too, that the ammonia content of venous blood draining an exercising forearm is elevated(3). Peripheral systemic blood ammonia has not been measured following voluntary muscular exercise. Studies were carried out in human and animal subjects to 1) determine if there is significant elevation of peripheral blood ammonia following voluntary muscular exercise or following convulsions induced by electro-shock or metrazol, and 2) correlate changes in blood ammonia with changes in ventilation following muscular exercise.

Methods. Alterations in blood ammonia in various types of muscular exercise have been studied as follows: 1) muscular exercise induced by electroshock therapy in humans or by metrazol in dogs, and 2) voluntary muscular exercise in normal human males (running). In both groups blood ammonia, pH, and plasma total CO_2 content were measured during a control period and at suitable intervals following exercise or convulsions as indicated in Fig. 1 through 4. Blood ammonia was analyzed by the method described by Bessman and Bessman(4). Minute respiratory volume was measured only on normal human subjects prior to, and following, voluntary muscular exercise. These measurements were carried out using a Tissot Gasometer. Studies of patients subjected to electroshock therapy were carried out under 2 conditions: 1) a group of 9 psychiatric patients who received only atropine as premedication and had violent generalized muscular contractions for approximately one minute (Group 1); and 2) 5 patients who received anectine, surital and atropine prior to electroshock therapy, thereby largely preventing motor aspects of the convulsions (Group 2). In 3 anesthetized dogs convulsions were induced by administration of 40-160 cc of metrazol in 1-3 cc increments at rate sufficient to produce severe muscular movements. Duration of convulsions by this method was 5, 25, and 30 minutes. Arterial blood was used for chemical analysis.

Results. Alterations in patients subjected to electroshock therapy: The data in Fig. 1 illustrate changes in blood ammonia, pH, and plasma total CO_2 in 9 psychiatric patients given electroshock therapy accompanied by marked convulsions (Group 1).† In 6 patients there was an elevation of blood ammonia, and decrease in pH and total CO_2 immediately following convulsions. In 2 of 3 other patients, blood drawn after 20 minutes showed ammonia levels which were similar to control values. Data shown in Fig. 2 illustrate minimal alterations in 5 patients who, due to premedication, did not have muscular convulsions following electroshock therapy (Group 2). In this group only one patient displayed a slight elevation of blood ammonia, and he had a

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† The calculated PCO_2 values in these patients before shock were 4-5 minutes Hg higher than normal. We have no explanation for this.

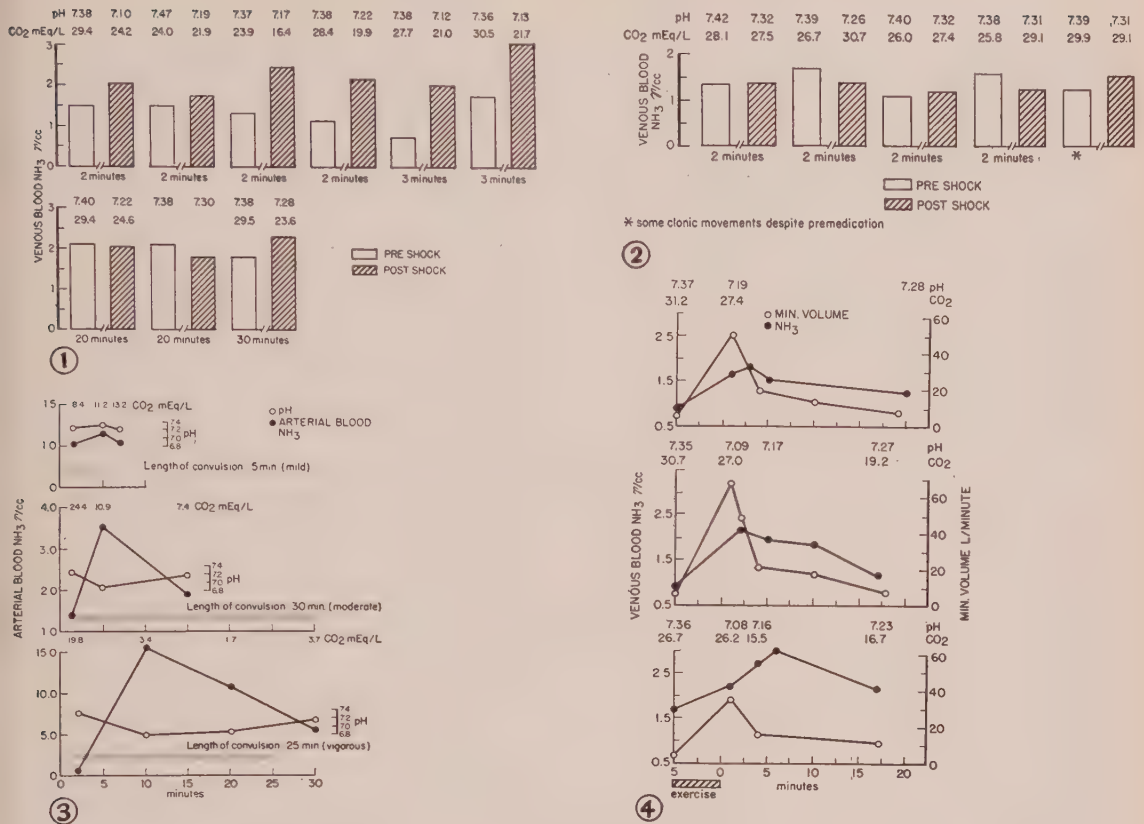

FIG. 1. Blood ammonia and acid-base alterations in 9 patients subjected to electroshock therapy accompanied by active convulsions.

FIG. 2. Blood ammonia and acid-base alterations in 5 patients subjected to electroshock in whom active convulsions were prevented by premedication.

FIG. 3. Blood ammonia and acid-base alterations in 3 dogs following metrazol convulsions.

FIG. 4. Blood ammonia and respiratory minute vol after severe exercise for 5 min. (human subjects).

small convulsion despite premedication.

Alterations in dogs given metrazol. There was a marked decrease in blood pH and plasma total CO_2 in dogs with convulsions following metrazol administration (Fig. 3). The rise in peripheral blood ammonia was also striking. Changes in blood ammonia, pH, and plasma CO_2 were greater than were measured in human subjects given electroshock therapy.

Changes in blood ammonia following muscular exercise: The data in Fig. 4 show the increase in blood ammonia at intervals up to 20 minutes following 5 minutes of voluntary strenuous muscular exercise in normal adult males. In the 3 subjects studied, the maximal elevation of blood ammonia was not reached until 2-6 minutes after the exercise was completed. The maximal elevation of blood ammonia could not be closely correlated with the maximal increase in minute volume. There was, however, a significant elevation of blood ammonia during the hyperpneic period following exercise. The blood pH and plasma CO_2 also decreased following muscular exercise.

Discussion. A combined metabolic and respiratory acidosis was observed following both artificially produced convulsions and voluntary exercise. These changes were, to some extent, a measure of magnitude of muscular activity. Of most interest to us were the small, but consistent, elevations of blood ammonia that occurred both after voluntary exercise and convulsive states.

The experimental production of metabolic or respiratory acidosis has been shown to cause some increase in blood ammonia in normal and Eck fistula dogs(5). This is of importance because most of the rises in blood ammonia were associated with decrease in blood pH. There were 2 observations, however, that suggested these changes in blood ammonia with exercise were of additional significance. Dogs subjected to mild voluntary exercise demonstrated elevation of blood ammonia in the post-exercise period, despite lack of significant change in blood pH. Also, a decrease in pH by acid infusion was associated with much smaller rise in blood ammonia than a comparable change in pH occurring after experimentally produced convulsions(5).

The source of the increase in blood ammonia would seem to be from within the muscles themselves as a result of deamination. Earlier work with isolated muscle suggests that ammonia formation is more prominent following the period of muscle fatigue than during the active period of contraction(6), and this is supported by the observed changes in blood ammonia in this study.

Although the respiratory stimulant, ammonia, was elevated following muscular exercise or active convulsive states, the actual significance of this observation is not entirely clear. The peak of increase in ventilation was immediate in the post-exercise period, but maximal elevation of blood ammonia did not occur until approximately 4 minutes post-exercise. Ammonia elevation which did occur, persisted to some extent after ventilation had returned to control values. The increase in cardiac output and oxygen consumption with muscle exercise also continues for a longer period than the hyperpnea. The possibility that ammonia plays some role in normal respiratory response to exercise is suggested by the consistent observation of increased blood ammonia following various forms of exercise studied.

Summary. 1. Peripheral blood ammonia concentrations were elevated following convulsions due to electroshock therapy in patients, convulsions in dogs due to metrazol, and active voluntary exercise in human subjects. 2. The possibility that increase in circulating blood ammonia may be a factor in hyperpnea of muscular exercise is discussed.

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1. Comroe, J. H., *Physiol. Rev.,* 1944, v24, 319.
2. Roberts, K. E., Thompson, G. F., III, Poppell, W. J., Vanamee, P., *J. Appl. Physiol.,* 1956, v9, 367.
3. Parnas, J. K., Mozolowski, W., Lewinski, W., *Biochem.,* 1927, v188, 15.
4. Bessman, S. P., Bessman, A. N., *J. Clin. Invest.,* 1955, v34, 622.
5. Lawrence, W., Jr., Jacquez, J. A., Dienst, S. G., Poppell, J. W., Randall, H. T., Roberts, K. E., *Surg.,* 1957, v42, 50.
6. Mozolowskl, W., Mann, T., Lutwak, G., *Biochem.,* 1931, v231, 290.

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Electromagnetic Blood Flow Meter Yielding a Base Line Without Interruption of Flow.* (24104)

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The latest modification of the electromagnetic flowmeter(1) constitutes an improvement over previous forms(2,3,4) in that it permits recording of blood flow in essentially freely moving conscious animals. But 2 problems remained to be solved to make it an instrument of unrestricted usefulness in physio-

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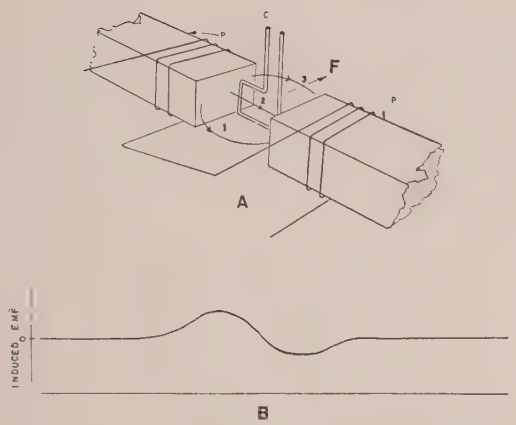

FIG. 1. A. Non-homogeneity of magnetic field depicted with exaggeration by flux lines 1, 2, 3. C: equivalent loop representing electrode circuit. F: direction of blood flow. P: pole pieces of magnet. B. Emf induced in coil C as a function of its position along the flow axis F.

logical research: 1) The instrument had to be made insensitive to the ever present 60 cps pickup so that the animal could move about and be handled during recordings of blood flow; 2) determination of zero base line had to be accomplished without interruption of flow and thus without auxiliary devices, such as plastic occlusion bags previously used(1,3) implanted in addition to flow meter unit. Both problems have now been solved by measures here described.

Methods. Elimination of the 60-cycle pickup has been accomplished by energizing the magnet with 400 or 1000 cps current and using an amplifier utilizing band pass filters sharply tuned to one of these frequencies. This obviates use of compensator(1,3) and permits injecting and otherwise handling the animal while recording of blood flow is in progress without noticeable disturbance of the record. In addition to freedom from interference, a second advantage is realized. The signal to noise ratio is considerably improved at higher frequency, as shown in Fig. 2 which shows records of blood flow in the carotid artery of a dog. Fig. 2A was taken at 60 cps and Fig. 2B at 400 cps. The reduction in noise at 400 cps is most clearly seen by inspection of sections of records during intervals when flow meter magnet is turned off *i.e.*, when flow record is absent. A frequency above 60 cps (200 cps) has previously been used with a sinusoidal field flow meter(5). Working at higher frequency increases the difficulty in obtaining the zero-flow base line without interruption of flow. This is due to the fact that transformer emf induced in flow meter output circuit is proportional to frequency of the magnetic field. This emf comes about as follows: the 2 pickup electrodes bridged by the artery can be considered as the secondary loop of a transformer. The primary coil is formed by the turns energizing the magnet. The primary and the secondary coils are linked by the magnetic field in the gap of the magnet. The above mentioned secondary loop can be symbolized by the one-turn loop C shown in Fig. 1A. Since the magnetic field lines are not strictly parallel, there is, in general, an emf induced in the coil C due to the magnetic field component which is perpendicular to the plane of the loop. This emf varies as the loop is moved parallel to its original orientation through the magnet gap parallel to the flow axis F. Fig. 1B shows the record of the phase and magnitude of the emf induced in coil C as it moves from position 1 in the gap to position 2. Curvature of the magnetic flux lines in Fig. 1A is greatly exaggerated to emphasize the change in direction of the magnetic field near the visible pole face as the coil is transferred from position 1 to position 3. This transfer is accompanied by phase reversal of the emf induced in C as shown in Fig. 1B. Between these two extreme positions, there is a position 2 in which the resultant magnetic flux perpendicular to area

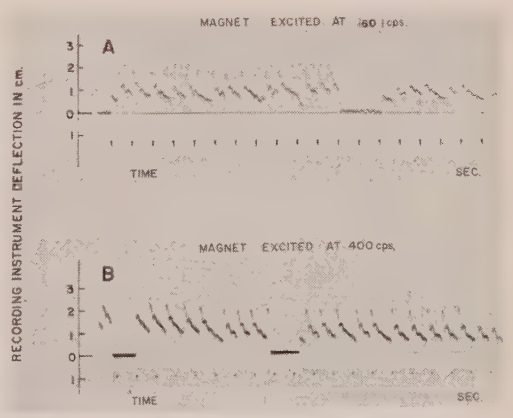

FIG. 2. Comparison of signal to noise ratios at 60 cps (Fig. 2A) and 400 cps (Fig. 2B).

of loop C is zero. At this critical point the transformer emf induced in C vanishes, and the output of the secondary loop C becomes independent of whether the magnet is on or off.

The preceding discussion assumes that the loop C is solely responsible for the induced emf which is to be eliminated. This is not the case. Part of the undesired emf is induced in the input leads connecting to loop C by the alternating magnetic stray field. This emf is balanced out along with the emf induced in the loop C by the positioning of the sleeve described above.

In the process of construction of an electromagnetic flow meter, the plastic sleeve containing the electrodes(1) is adjusted by movements parallel to F until the critical position is reached in which the signal voltage detected between electrode leads remains unchanged as the magnetic field is turned on and off. This adjustment is carried out preferably with the flow meter submerged in saline, although smaller units can be satisfactorily adjusted in air while electrodes are connected by a metal bar parallel to their axis or by a filling of Wood's metal.†

After completion of this adjustment, the sleeve is polymerized in the plastic body of the flow meter, as previously described(1), and the sensing unit is ready for use without the need for an occlusion bag for the establishment of a base line. The latter can now be established by turning off the electromagnet.

De-energizing the magnet to ascertain the base-line has previously been used in connection with constant magnetic field flow meters (6,7). Subsequently, a square wave current has been used to energize the magnet(4) in order to obtain during the constant current interval of the square wave conditions similar to those which prevail in a constant magnetic field. This arrangement also permits the establishment of the base line by de-energizing the magnet. The method herein described accomplishes the same by energizing the magnet

† To facilitate the zero adjustment, it is helpful to slightly distort the magnetic field by making one pole piece somewhat thinner than the other.

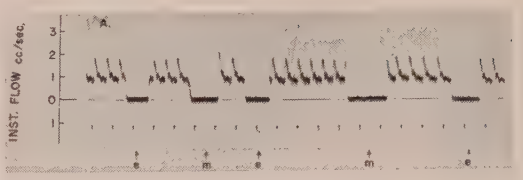

FIG. 3. Comparison between base lines obtained by interruption of flow (m) and switching off magnetic field (e).

with an ordinary sinusoidal current and basing the avoidance of a signal voltage at zero flow on a different principle which was described above. This obviates the need for complex circuitry used with the square wave device(4). A standard 4 stage amplifier with transformer input utilizing two Z-8324 EEco plug-in amplifier units proved quite satisfactory.

The effectiveness of this device is shown in Fig. 3. This record shows a comparison between base lines established by interruption of blood flow by means of a device compressing the artery (m) and by turning off the magnet without interruption of flow (e). The two base lines can be considered to be identical for all practical purposes. Periodic checks of the stability of the base line established in this fashion reveal that it remains unchanged in the course of many hours of continuous operation.

Summary and conclusion. The necessity of implanting an occlusion bag to secure a base line at zero flow has been eliminated. This has been accomplished by placing the pick-up electrodes in the magnetic field to eliminate the transformer emf induced in the input lead circuit. As the sleeve is moved parallel to flow axis a point is found at which the a.c. voltage between electrodes vanishes regardless of whether the magnet is on or off. Thus, the baseline is obtained by merely switching off the magnet. The circuit has been improved to a point where the animal can be handled without interference with the recording of blood flow.

1. Kolin, A., Assali, N., Herrold, G., Jensen, R., *Proc. Nat. Ac.*, 1957, v43, 527.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 235.

3. ———, *Rev. Sc. Instr.*, 1952, v23, 235.

4. Dennison, A. B., Spencer, M. P., Green, H. D., *Circ. Research*, 1955, v3, 39.

5. Richardson, A. W., Dennison, A. B., Green, H. D., *Circulation,* 1952, v5, 430.
6. Wetterer, E., *Z. Biol.,* 1937, v98, 26.
7. Kolin, A., *Am. J. Physiol.,* 1938, v122, 797.

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Postnatal Histogenesis and Endocrine Function of Abnormal Testes in the AxC Rat. (24105)

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Deringer and Heston have described the relatively frequent occurrence of multiple abnormalities in the genitourinary tract of the AxC rat(1). In the male the anomalous pattern includes a total absence of kidney and ureter on one side frequently associated with presence on the same side of an atrophic testis which lacks a vas deferens or epididymis. In addition the seminal vesicle, but not the coagulating gland, is absent on the involved side. In this strain of rats varying patterns of this anomalous picture are observed in approximately 20% of all male animals studied(1). Similar patterns of genitourinary abnormality have been described in man by Whitehorn (3) and Longo and Thompson(4).

We wish to report our observations on postnatal development of the abnormal testis in the AxC rat. These studies have indicated that during prepuberal period the testis which is destined to be atrophic in adult life is morphologically and histochemically indistinguishable from the normal testis of comparable age. With onset of puberty the abnormal testis presents its initial deviation from normal developmental pattern, namely complete failure of tubular maturation and spermatogenesis.

Materials and methods. The rats were derived from a highly inbred strain previously described by Heston and Deringer(1). They were kept in metal cages in constant-temperature animal quarters maintained between 76 and 80°F. They were fed Purina stock diet *ad libitum* and provided constant supply of tapwater. Pregnant animals were isolated for delivery and date of birth of all litters recorded daily. All animals were sacrificed by ether at indicated age. Testes which could be expected to be atrophic were identified by occurrence on the same side as the observed absence of vas deferens, kidney, ureter and seminal vesicle. This was most commonly on the right side. The contralateral testis was used as normal control tissue since no instance of bilateral testicular failure has occurred in this colony. All tissues were promptly weighed and fixed for histochemical analysis. The following fixatives and staining methods were employed: I. Bouin's solution; for morphological study, glycogen and mucopolysaccharide. II. Cold alcohol, 80%; for alkaline phosphatase; III. Barnett and Bourne's solution(5); for ascorbic acid; IV. Baker's Formaldehyde(6); for total lipids, cholesterol and silver impregnation; V. Mallory's trichrome stain as modified by Crossman(7); VI. Lillie's allochrome stain(8); VII. Del Hortega's(9) or Gridley's(10) silver impregnation; VIII. Weigert's resorcin-fuchsin(11); IX. McMannus' P.A.S. for glycogen(12) with takadiastase digestion at 37°C for control; also with hyaluronidase digestion to eliminate reaction with some muco-polysaccharide; X. Gomori's method as modified by Kabath and Fursth(13) for alkaline phosphatase; XI. Metachromatic reaction with Azur B and Toluidine Blue O (0.055%, pH 6 and pH 4) and P.A.S. for acid mucopolysaccharides; XII. Sudan (III, IV and Black) for lipids; XIII. Schultz's(14) and Brunswick's methods(15) for cholesterol and cholesterol esters; XIV. Dean and Morse's method(16) for ascorbic acid. Thirty-five abnormal specimens were selected to cover the prepuberal as well as postpuberal and adult period (Table I). In addition, tissues from 65 normal animals at different ages were studied for com-

TABLE I. Weight and Position of Normal and Abnormal Testes in 35 AxC Rats at Various Ages.

Age, days	Abnormal testis		Normal testis	
	Wt, mg	Position	Wt, mg	Position
3	3	abdominal	3	abdominal
5	6.5	"	6.7	"
8	13	"	13	"
9	18	"	16	"
10	19	"	19	"
19	63	"	62	"
24	94	low abd'nal	96	low abd'nal
"	100	" "	100	" "
30	184	abdominal	194	scrotal
38	182	"	330	"
44	440	scrotal	550	"
180	230	abdominal	1250	"
"	500	scrotal	1600	"
"	450	"	1440	"
"	98.2	"	1450	"
"	170.5	"	1250	"
"	217.5	"	1560	"
"	192	abdominal	1410	"
"	375	scrotal	1390	"
"	490	"		*
"	255	"		*
"	†	abdominal		*
"	320	scrotal		*
"	380	"		*
"	†	abdominal	1350	*
"	470	scrotal	1235	*
"	570	"	1320	*
"	660	"	1280	*
"	540	"	1415	*
"	500	"	1310	*
"	†	abdominal	1290	*
"	520	scrotal	1345	*
"	540	"	1545	*
"	430	"		*
"	370	"		*

* Scrotal testis extirpated 6 to 11 mo before.
† Hydrotestis.

parison. In 16 abnormal adult animals, the normal testis had been surgically removed 6 months before autopsy to determine whether the remaining atrophic testis could maintain the prostate and seminal vesicle of the host. To ascertain whether or not absence of vas deferens plays a critical role in abnormal development of the anomalous testis, one vas deferens was surgically removed from each of 10 normal animals when they were 22 to 25 days old (Table II). This operation was performed under magnification so that no traumatic interference with testicular blood supply would result. These animals were then sacrificed at 61 to 64 days of age and the testes of operated side and of unoperated side were then compared by technics listed above.

Results. Histological and histochemical review of the chronologically distributed specimens permits one to reconstruct the histogenetic process ultimately leading to the pathological state found in the adult. Thus, close comparison of specimens of normal and abnormal side from rats up to 30 days of age reveals essentially the same picture in both sides. Up to this stage, there is a normal progression in either testis and it is noteworthy that no significant discrepancy in testicular weight is noted. In earlier specimens up to 10 days of age (Fig. 11) the tubular structures lack a lumen and are represented by solid cords consisting of two types of cells: (a) small cells with a sharply defined nuclear membrane, coarse chromatin granules and a distinct nucleolus; they are located in the periphery of the cord and exhibit weakly positive nuclear reaction for alkaline phosphatase and negative cytoplasmic reaction for alkaline phosphatase, ascorbic acid and lipids, and (b) larger cells, oval or round, with nuclei containing 2 or 3 nucleoli and presenting a negative reaction for lipids and ascorbic acid but positive alkaline phosphatase response in both nucleus and cytoplasm. The latter cells become less abundant as development proceeds and are nearly absent by 9th day. The cellular cords lack a definite limiting membrane but are closely surrounded by interstitial connective tissue. The latter is made up of a network of thin reticular fibres, many mesenchymal cells and immature fibroblasts which are dispersed in a highly eosinophilic amor-

TABLE II. Effect of Extirpation of Vas Deferens on Testis Weight of 12 Rats.

No.	Age at extirpation of vas deferens (days)	Age at autopsy (days)	Testicular wt (mg) Left	Right
1	22	64	1007	1005
2			710	730
3			1120	1112
4			550	520
5			1130	1115
6	25	61	1100	1150
7			1000	1000
8			1050	1000
9			1100	1050
10			1030	1020
11	Not operated	64	1350	1008
12			1000	990

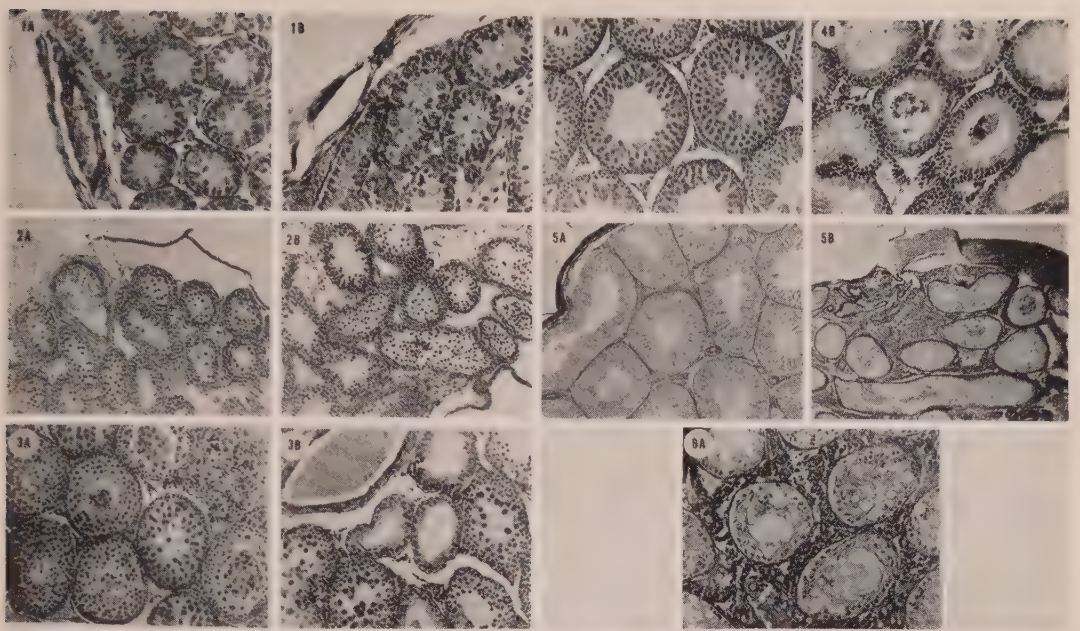

FIG. 1. Testes from 8-day-old AxC rat (× 250). a. Normal testis. b. Testis from abnormal side.

FIG. 2. Testes from 19-day-old AxC rat (× 125). a. Normal testis. b. Testis from abnormal side.

FIG. 3. Testes from 30-day-old rat (× 125). a. Normal testis. b. Testis from abnormal side.

FIG. 4. Testes from 38-day-old AxC rat (× 125). a. Normal testis. b. Testis from abnormal side.

FIG. 5. Testes of adult AxC rat. a. Normal testis. b. Abnormal testis (× 70).

FIG. 6. Abnormal testis of adult AxC rat (× 125).

phous ground substance which stains a deep blue with Azur B and is strongly positive to P.A.S. In the interstices there are also found small groups of oval cells which resemble adult Leydig cells, and possess acidophilic cytoplasm which stains positively for alkaline phosphatase, lipids and cholesterol.

Between 19 and 24 days the respective testis weights from each side continue to be equal. The tubular diameter increases and there are clear lumina formed (Fig. 2). The tubular wall is well formed and the basal membrane responds positively to P.A.S. and to stain for alkaline phosphatase. The surrounding reticular fibres have now become highly condensed. The tunica propria consisting of a reticular net and numerous fibroblasts are noted. Within the tubules, the more embryonic cells are now replaced by distinct Sertoli cells, spermatogonia, and spermatocytes. These elements in both testes present an entirely normal appearance and stain positively for lipids, ascorbic acid and alkaline phosphatase. The interstitial spaces are now larger and present numerous fibroblasts in varying stages of maturation. Immature and mature Leydig cells appear entirely normal in both testes and are now enmeshed in a less dense reticular network.

In the 30- to 38-day period distinct weight differences are noted between testis of abnormal and normal side and the pathological features of the smaller gonad clearly emerge (Fig. 3 and 4). The essential deficiency is a progressive failure of normally evolving tubular maturation and spermatogenic function, whereas the interstitial cell development proceeds normally. After 44 days of age and on into advanced adult life the deficient gonad presents all features of the definitive syndrome (Fig. 5, 6). There is total failure of spermatogenic function and the atrophic tubules present a distinctly thickened wall of reticular, elastic, and collagen fibres. The abun-

dant homogeneous intertubular amorphous substance which stains red with eosin, light blue with azure, and pink with P.A.S. also resists hyaluronidase and diastase digestion. Hence it appears to be comparable to the interstitial fluid found normally in immature and puberal testes. Leydig cells are present in normal numbers and present all normal histochemical reactions for cells of this type. However, both vascular sclerosis and proliferation of normal fibroblasts are seen.

The hormonal functional capacity of Leydig cells of the atrophic testis is also shown to be normal by lack of atrophy of the prostate or seminal vesicles in 16 adult rats which had undergone extirpation of their normal testis 6 months before autopsy.

Moreover, there was no effect of vas deferens extirpation upon subsequent development of the normal testis of rats of this strain. This indicates that absence of the vas deferens in the case of anomalous testis is probably not a critical factor in development of the abnormal state.

Specificity of the various technics used, has been extensively discussed(8,17,18,19,20). It is especially noteworthy that by all these histochemical criteria the uninvolved testis in the AxC rat may be considered entirely normal. Moreover, the abnormal testis shows no appreciable deviation from the normal pattern of development until the pubertal stage is reached. It would appear then that this anomaly represents a delayed somatic expression of a genetically determined defect.

The anatomical position of each of the testes of each animal was recorded at autopsy. Since the inguinal canal in the rat remains open throughout life these data are of dubious significance except as an indication that scrotal or abdominal position of the testes was totally unrelated to the histological picture in any given case.

Association of a testicular defect with such other defects as absence of kidney, ureter, vas deferens, and seminal vesicle, reflects multiple manifestations of anomalous embryonogenesis arising from defective tissue along the entire mesonephric ridge as well as a consequent failure of normal induction of development of the kidney from the metanephric anlagen. Further analysis of the embryological course of events in the AxC rat should aid in elucidation of normal developmental interrelationship in this region.

Summary. The male AxC rat exhibits a genetically-determined anomalous development of the genitourinary apparatus including absence of one kidney, ureter, vas deferens, and seminal vesicle associated with testicular hypoplasia on the same side. Detailed histochemical and histological analysis of the postnatal development of the defective testis reveals that this testis can not be differentiated from the normal testis until puberty, at which time it exhibits a total failure of tubular maturation and spermatogenic function. Interstitial cells, however, continue to develop normally and exhibit normal endocrine function and removal of the normal testis does not lead to atrophy of the prostate or seminal vesicle. Surgical extirpation of the vas deferens during the prepuberal period does not lead to any alteration in subsequent development of the normal testis. It may be concluded that this instance of testicular hypoplasia represents a genetically determined defect with delayed somatic manifestation.

1. Deringer, M. K., Heston, W. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 312.

3. Whitehorn, C. A., *J. Urol.*, 1956, v72, 685.

4. Longo, V. J., Thompson, G. J., *ibid.*, 1952, v68, 63.

5. Barnett, S. A., Bourne, G., *J. Anat.*, 1940, v75, 251.

6. Baker, J. R., *Quart. J. Microscopical Sci.*, 1945, v85, 1.

7. Crossmon, G., *Anat. Rec.*, 1937, v69, 33.

8. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry*, Blakiston Co., N. Y., 1954.

9. del Rio Hortega, P., *Bol. Soc. Esp. Hist. Nat.*, 1921, v21, 364.

10. Gridley, M. F., *Am. J. Clin. Path.*, 1951, v21, 897.

11. Weigert, C., *Centralbl. Allg. Path.*, 1898, v9, 289, in McClung's Handbook of Microscopical Technique, Paul Huber, Inc., N. Y., 1950.

12. McManus, J. F. A., *Nature*, 1946, v158, 202.

13. Kabath, E. A., Fursth, J., *Am. J. Path.*, 1941, v17, 313.

14. Schultz, A., *Zentralbl. All. Path. Anat.*, 1924, v35, 314, in Glick's Techniques of Histo and Cyto-

chemistry, Interscience Publishers, N. Y., 1949.
15. Brunswick, O., *Z. Wiss. Mikr.*, 1922, v39, 316.
16. Deane, H. W., Morse, A., *Anat. Rec.*, 1948, v100, 127.
17. Lison, L., *Histochimie et Cytochimie Animals*, Gauthier-Villars, Paris, 1953.
18. Gomori, G., *Microscopic Histochemistry*, Univ. of Chicago Press, 1952.
19. Pearse, A. G. E., *Histochemistry: Theoretical and Applied*, Little, Brown and Co., Boston, 1954.
20. ———, *Ciba Foundation Colloquia on Endocrinology*, 1952, v4, 1.

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Anaphylaxis in the Mouse Produced with Soluble Complexes of Antigen and Antibody.* (24106)

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The concept recently expressed by Germuth(1) and Germuth and McKinnon(2) that lesions of hypersensitivity diseases may result from local accumulation of soluble complexes of antigen (Ag) and antibody (Ab) derived from the circulating blood is a highly significant contribution toward an understanding of the mechanisms of immunologic tissue injury. The proposition that soluble Ag-Ab complexes initiate development of allergic lesions is indirectly supported by many earlier observations, principally concerned with temporal relationships of development of lesions to blood clearance of Ag and appearance of circulating Ab. Production of anaphylaxis in the guinea pig with soluble complexes of Ag and Ab(2) provides strong support to this proposition. Of the observations important to development of the concept were those of Hawn and Janeway(3) and Germuth, Pace and Tippett(4) that acute lesions of "serum sickness" in rabbits develop while Ag is still present in circulating blood, and heal only after free circulating Ab appears, and those of Talmage, Dixon, Bukantz and Dammin(5) that a rapid "immune phase" of Ag elimination by Ab always precedes appearance of free Ab in the circulation. These findings are consistent with the report of von Pirquet and Schick(6) that clinical symptoms of serum sickness in humans commonly developed a week or more before appearance of any precipitating Ab in blood, and often subsided before Ab disappeared. Proportions of various types of antibodies present in soluble complexes of Ag and Ab reported to occur in actively sensitized rabbit following large doses of bovine serum albumin (BSA)(2,5) are not known. Therefore, it is not possible to predict the respective roles that precipitating and non-precipitating Ab may play in development of allergic reactions through the agency of their soluble complexes with Ag. However, recent observations that soluble complexes of BSA and anti-BSA rabbit Ab, presumed to be composed largely of complexes of precipitating Ab and Ag, produce anaphylaxis in guinea pigs(2) and in mice(7) indicate that precipitating Ab is important in pathogenesis of allergic reactions by this mechanism. The observation of Kabat and Benacerraf(8) that non-precipitating Ab is equally as effective as precipitating Ab for producing passive anaphylaxis in the guinea pig favors the prediction that complexes of non-precipitating Ab and Ag would likewise produce anaphylaxis. That non-precipitating Ab can cause serum sickness in humans appears to have been well established by Karelitz(9). The concept that a non-precipitating Ab may be important in diseases such as rheumatic fever has been tested by Weiser (unpublished), Kuhns and McCarty(10), Quinn(11) and Rejholec(12). Rejholec (12) observed that rheumatic fever subjects show a greater capacity to form incomplete Ab against brucella vaccine than do non-rheumatic subjects.

Materials and methods. Unless otherwise

* This investigation supported in part by Washington State Heart Assn.

TABLE I. Anaphylaxis in Mouse Produced with Soluble BSA Anti-BSA Complexes Formed in Various Regions of Ag Excess.

Challenge preparation	Vol of challenge dose, ml	Ab N in challenge dose, mg	Deaths*	% mortality	Symptoms of survivors† Severe	Mild
Complex 8x Ag excess	.4	.462	28/34	82	6	
Idem 8x	.75	"	27/31	87	4	
" 20x	.4	"	17/21	81	4	
" 40x	.4	"	15/21	72	6	
" 60x	.4	"	12/30	40	14	4
" 180x	.75	"	5/32	16	20	7
" 180x (converted from 8x Ag excess)	.75	"	0/ 6	0	5	1
Ag 200x 5 min. before challenge with complex 8x Ag excess	.4	"	6/ 6	100		
Controls						
Complex 8x Ag excess + normal rabbit serum containing 44 mg protein	.4	.462	25/27	93	2	
Ag 200x	.75		0/ 7	0	no shock	
Anti-BSA serum	.75	1.30	0/20	0	*Idem*	
Ag 8x + normal rabbit serum	.4		0/ 8	0	"	
Supernatant fluid from zone of equivalence	.6		0/12	0	"	
BGG (2.5 mg) in anti-BSA serum	.4	.462	0/ 8	0	"	
Anti-BSA serum + EL (75 mg)	.5	"	0/12	0	"	

* Denominator represents total No. of animals; numerator, the No. that died of shock. Death occurred 15 to 35 min.

† Severe and mild shock.

Ag = antigen; Ab = antibody; BSA = bovine serum albumin; Ab N = antibody nitrogen; EL = egg white lysozyme. An equivalent dose of Ag = 0.0424 mg AgN.

stated, materials and methods were similar to those employed in previous investigations(7). Soluble complexes of BSA† and anti-BSA were produced in various regions of Ag excess ranging from values of 8 "equivalent doses" of Ag to 180 equivalent doses of Ag. An equivalent dose of Ag is that amount of Ag present in a precipitate at equivalence for any given amount of Ab. The antisera were heat-inactivated at 56°C for 30 minutes immediately before use. Each preparation was made by adding heat-inactivated anti-BSA rabbit serum to a solution of BSA in physiological saline buffered at pH 7.0 with M/100 Sorenson's phosphate buffer. This phosphate buffered saline (PBS) was used as a diluent. The mixtures were incubated at 37°C for 20 minutes and centrifuged to remove any small amount of particulate matter present. In certain experiments soluble Ag-Ab complexes were prepared as described by adding concentrated solutions of Ag to precipitates formed in the zone of equivalence. Bovine gamma globulin (BGG)† and crystalline egg white lysozyme (EL)† were used in certain control preparations. The various challenge and control preparations were injected into the tail vein. Mice were distributed uniformly among various groups with respect to age, weight and sex. They were from 6 to 10 weeks old and ranged in weight from 23 to 30 g.

Results. In the first experiment groups of mice were challenged with soluble BSA anti-BSA complexes formed in various regions of

† Lots of bovine gamma globulin and crystalline egg white lysozyme were obtained from Armour and Co., Chicago, Ill. as was crystalline bovine serum albumin.

TABLE II. Anaphylaxis in Mouse Produced with Soluble BSA Anti-BSA Complexes Derived from BSA Anti-BSA Precipitates.

Challenge preparation	Vol of challenge dose, ml	Ab N in challenge dose, mg	Deaths	% mortality	Symptoms of survivors (severe)
Complex dissolved in 20x Ag excess	.6	.462	9/18	50	9
Idem, 180x	.6	.462	12/18	67	6

Ag excess. The results are presented in Table I. All control preparations devoid of Ag-Ab complexes were completely innocuous. The complexes formed in the regions of 60x and 180x Ag excess produced a significantly lower mortality than was observed with complexes formed in the regions of 8x, 20x and 40x Ag excess. Although there was a general trend of increasingly lower mortality over the range from 20x Ag excess to 180x Ag excess, the first significant reduction occurred at the level of 60x Ag excess. Despite the fact that differences in extent of shock observed among survivors may be of questionable significance it is notable that the only survivors which showed mild shock were in the groups that received the complexes formed in the regions of 60x and 180x Ag excess. It was observed that the anaphylactigenicity of the complex which was initially allowed to form in 8x Ag excess and then converted to 180x Ag excess by addition of Ag was similar to that observed for the complex initially formed in 180x Ag excess. This suggests that the large soluble complexes initially formed in regions of low Ag excess are readily reversible to simpler complexes in regions of extreme Ag excess. As expected, the intravenous injection of 200 equivalent doses of Ag 5 minutes prior to challenge with the complex formed in 8x Ag excess afforded no protection against shock.

Since it seemed possible that the nature of the soluble Ag-Ab complexes initially formed in the region of Ag excess might differ from soluble Ag-Ab complexes derived from precipitates formed in the equivalence zone, an experiment was next conducted with soluble complexes prepared by solubilizing such precipitates with excess Ag. An equivalent dose of BSA was added to anti-BSA rabbit serum, mixed, incubated for 1 hour at 37°C and the precipitate washed 3 times in PBS at 4°C. The precipitate was dissolved by agitation and incubation for 1 hour at 45°C in a concentrated solution of Ag containing 20 equivalent doses of Ag. The minute amount of precipitate remaining was removed by centrifugation and to one-half of the supernatant was added a concentrated solution of Ag to give a final solution containing 180 equivalent doses of Ag. An equal volume of PBS was then added to the 20x Ag solution and both solutions of the complexes were incubated at 45°C for 1 hour. They were stored in the refrigerator for 3 hours and warmed to 37°C immediately before injection into mice.

The data presented in Table II show that the complexes in both mixtures were essentially equivalent in their anaphylactigenicity. These results were in marked contrast to those of the first experiment in which it was observed that the complex formed in the region of 180x Ag excess and the complex converted to 180x Ag excess from 8x Ag excess were both markedly less anaphylactigenic than the complex formed in the region of 8x Ag excess.

Since it was thought that the use of unheated sera or physiological saline instead of PBS might alter the results, experiments were conducted using these materials. The results presented in Table III indicate that the anaphylactigenicity of the complexes was not lessened by the use of heated sera and physiological saline. They do not exclude the possibility that complexes formed in unheated sera are more anaphylactigenic than complexes formed in heated sera.

Discussion. Reasons to account for variations in the anaphylactigenicity of the various complexes are not readily apparent. There is suggestive evidence that large complexes derived from precipitates by solubilization with excess Ag have higher bond strength and are not as readily reversed to simpler complexes

TABLE III. Anaphylaxis in Mouse Produced with Soluble BSA Anti-BSA Complexes Formed in Various Regions of Ag Excess Using Unheated Antisera and Physiological Saline and PBS as the Diluents.

Diluent	Challenge preparation	Challenge dose, ml	Ab N in challenge dose, mg	Deaths	% mortality	Symptoms of survivors Severe	Mild
Saline	Complex 8x Ag excess	.5	.462	12/12	100		
"	*Idem*, 180x	"	"	1/12	8	8	3
PBS	" 8x	"	"	10/12	83	2	
"	" 180x	"	"	1/12	8	7	4

in extreme Ag excess as are soluble complexes initially formed in the region of low Ag excess(13,14,15). Perhaps it is only when complexes are allowed to form in extreme Ag excess that relative pure preparations of the simplest theoretical complex Ag-Ab-Ag are achieved and maintained.

There is limited information on the size of various soluble Ag-Ab complexes in protein anti-protein systems maintained in various regions of Ag excess and no definitive information on velocities of forward and backward reactions among these complexes which attend changes in Ag concentration. Singer and Campbell(16) observed that when a precipitate formed by BSA and salt-fractionated anti-BSA rabbit globulin was solubilized in low Ag excess the complexes were heterogeneous and very large. In contrast the complexes solubilized in regions of large Ag excess were smaller and rich in a complex of the apparent molecular composition Ag-Ab-Ag in linear array.

Additional factors which may influence rate of formation, size and composition of soluble complexes for any given Ag-Ab system include temperature(17), pH(18,19), salt concentration and ionic strength(14,18), and the presence of other macromolecular substances such as the various components of complement(20.21,22). Many of the problems concerned with determining the nature of such complexes have been discussed(14,17,23,24).

Despite the observation that the C'2 and C'3 components of complement are low in the mouse(25), it seems possible that in the present experiments mouse complement contributed to anaphylaxis by being fixed to and activated by the injected soluble Ag-Ab complexes. This possibility is especially attractive in view of the recent observations of Weigle and Maurer(21,22) that soluble complexes of BSA and anti-BSA rabbit Ab maintained in various regions of Ag excess fix decreasing amounts of complement with increasing Ag excess.

If complement is involved in the anaphylactigenic action of soluble Ag-Ab complexes its role may be 2-fold; that of precipitating with the complexes(20,21,22) and of being activated by them.

We wish to propose the hypothesis that anaphylactigenic activity of soluble BSA anti-BSA complexes for the mouse is dependent upon their capacity to react with complement.

Summary. 1) Soluble BSA anti-BSA complexes formed in regions of extreme antigen excess are less anaphylactigenic for the mouse than similar complexes formed in regions of low antigen excess. 2) The hypothesis is advanced that anaphylactigenic activity of these complexes for the mouse is dependent upon their capacity to react with complement.

1. Germuth, F. G., Jr., *J. Exp. Med.*, 1953, v97, 257.

2. Germuth, F. G., Jr., McKinnon, G. E., *Bull. Johns Hopkins Hosp.*, 1957, v101, 13.

3. Hawn, C. V., Janeway, C. A., *J. Exp. Med.*, 1947, v88, 571.

4. Germuth, F. G., Jr., Pace, M. G., Tippett, J. C., *ibid.*, 1955, v101, 135.

5. Talmage, D. W., Dixon, F. J., Bukantz, S. C., Dammin, G. J., *J. Immunol.*, 1955, v67, 243.

6. von Pirquet, C. F., Schick, B., *Serum Sickness*, 1905, Williams and Wilkins Co., 1951.

7. Tokuda, S., Weiser, R. S., *Science*, 1958, v127, 1237.

8. Kabat, E. A., Benacerraf, B., *J. Immunol.*, 1949, v62, 517.

9. Karelitz, S., *Ann. N. Y. Acad. Sci.*, 1949, v52, 705.

10. Kuhns, W. J., McCarty, M., *J. Clin. Invest.*, 1954, v33, 759.

11. Quinn, R. W., *J. Immunol.,* 1956, v76, 246.

12. Rejholec, V., *Ann. Rheum. Dis.,* 1957, v16, 23.

13. Kleinschmidt, W. J., Boyer, P. D., *J. Immunol.,* 1952, v69, 257.

14. Johnson, P., Ottewill, R. H., *Faraday Soc. Discussions* #18, 1954, 327.

15. Weigle, W. O., Maurer, P. H., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 371.

16. Singer, S. J., Campbell, D. H., *J. Am. Chem. Soc.,* 1952, v74, 1794.

17. ———, *ibid.,* 1955, v77, 3499.

18. Marrack, J. R., Grant, R. A., *Brit. J. Exp. Path.,* 1953, v34, 263.

19. Singer, S. J., Campbell, D. H., *J. Am. Chem. Soc.,* 1955, v77, 3504.

20. Morton, J. I., Deutsch, H. F., *Arch. Biochem. and Biophys.,* 1956, v64, 26.

21. Weigle, W. O., Maurer, P. H., *J. Immuol.,* 1957, v79, 211.

22. ———, *ibid.,* 1957, v79, 223.

23. Becker, E. L., *J. Immunol.,* 1953, v70, 372.

24. Goldberg, R. J., Williams, J. W., *Faraday Soc. Discussions* #13, 1953, 224.

25. Muschel, L. H., Muto, T., *Science,* 1956, v123, 62.

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Development of Interarterial Intercoronary Anastomoses by Arteriovenous Fistula Between Pulmonary Artery and Left Atrium.* (24107)

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Observations made on man in open heart surgery have suggested the likelihood of existence of rich intercoronary networks in patients with congenital cyanotic heart disease (Tetralogy of Fallot). The possibility that inter-coronary anastomoses developed in response to cyanosis suggested the following study.

Method. Selected mongrel dogs 15-25 kg in weight were used. Weak or sick animals were rejected. All animals were anaesthetized with 3% sodium nembutal (30 mg/kg body wt.) and placed on a cam type respirator utilizing compressed air. Clean but not sterile technic was employed. A left-sided thoracotomy was performed and the 4th rib was removed. The pericardium was opened parallel to the phrenic nerve so as to display the main trunk of the pulmonary artery and the left auricle. A side to side arteriovenous anastomosis 10-17 mm in length was made in each animal between the pulmonary artery distal to the semilunar valves and the left atrium. The thoracic cavity was closed in layers and 500,000 units crystalline penicillin was given IM for 2 days. Systemic blood for sampling of oxygen content and saturation was obtained by direct cut-down upon the femoral artery. Determinations were by the method of Van Slyke and Neill(1). All animals tolerated the procedure well. Postoperative care consisted of routine kennel rations and a mandatory exercise period of 15 minutes each day for every animal. At varying periods (2 days to 14 weeks) after operation the dogs were sacrificed and the pattern of coronary arteries was studied by corrosion digestion preparations(2). An earlier study of the architecture of coronary arteries in 50 normal dog hearts, using the same injection-corrosion method, served as an adequate control.

Observations. Results are summarized in Tables I and II. With the exception of one animal that died 2 days after operation and in which the shunt was 16 mm in circumference, no dog appeared in acute respiratory distress. Subsequent experiments utilized a fistula no larger than 10-12 mm in circumference. In these animals, including several not reported at this time, there has been no postoperative death. One animal showed signs of dyspnea on exertion. The exercise

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TABLE I. Experimental Study.

Wt, kilos	Circumference of shunt (mm)	Wk post surgery at exam.	Intercoronary anastomoses between branches of left coronary arteries	Anastomoses present between right and left coronary arteries	Location of anastomoses between right and left coronary artery when present
18	16	2 days	Present	None	None demonstrated
16	15	10 "	"	Present	Region of crux, dorsal surface of heart
20	12	2 wk	"	"	Auricular anastomoses
15	12	3 "	"	"	Dorsal surface of heart
16	10	6 "	"	None	None demonstrated
15	10	8 "	"	Present	Dorsal surface of heart
15	12	14 "	"	"	*Idem*

TABLE II. Control Study, 50 Dogs.

Body wt, kg	Procedure	Intercoronary anastomoses present between branches of left coronary artery	Intercoronary anastomoses present between branches of right and left coronary arteries
5–25	Injection-corrosion study of coronary circulation using vinyl plastic mass at post-mortem	Arterial in size 30% Finer, small vessels 40%	0

tolerance of all other animals remained unimpaired or had adjusted. Systemic arterial oxygen saturation in the reported animals varied between 60 and 88% with an average value of 80%.

At post-mortem the shunt was patent in all cases. The diameter in every specimen was found to be reduced from the dimension recorded at the original operation.

Study of the vinyl plastic injection casts of the coronary artery distribution in the hearts of normal dogs (5-25 kg body weight) demonstrated that intercoronary anastomoses were regularly to be found between the branches of the left coronary artery.

Communications of large size were found in 30% of preparations between left anterior descending artery and branches of left circumflex coronary artery. In a further 40% of specimens fine networks of communicating vessels ramified about the cardiac apex and terminal branches of left anterior descending artery. In not a single injection-corrosion cast was an intercoronary interarterial anastomosis demonstrated between right and left coronary artery in a normal dog heart. In these studies no intercoronary anastomoses were found in region of the sino-atrial node.

Following surgical production of an arteriovenous fistula the plastic corrosion preparations in 5 of 7 animals clearly demonstrated the presence of an intercoronary communicating channel between branches of right and left coronary artery in animals sacrificed at intervals of 10 days to 14 weeks following surgery. These channels were arterial in size. In *every* case intercoronary anastomoses were present between branches of the left coronary artery. In all studies these channels were arterial in size.

Data relating to venous and arterial oxygen saturation, hemoglobin and hematocrit adjustments, changes in pH of systemic blood and variations in atrial pressure and in coronary flow are under investigation.

It is noteworthy that in no revascularising procedures examined in this laboratory aimed at creating intercoronary communications, utilizing the same vinyl plastic injection mass, has it been possible to demonstrate objectively intercoronary arterial anastomoses of the size observed in this study, nor with the same celerity. Only following acute total ligations of the circumflex or left anterior descending cor-

onary artery, with survival of the animal, have comparable collateral communications been observed.

Discussion. These observations may have significance in obliterating coronary artery disease in man. The essential role of anoxemia as an underlying stimulus for development of interarterial coronary anastomoses was indicated by Zoll and Schlesinger(3). Eckstein(4) later reported production of intercoronary anastomoses as a result of chronic anemia. Our injection-corrosion study of human hearts at autopsy confirmed those observations. The results presented in Table I suggest that not only do the "new" channels (doubtless always present potentially) provoked by operation function in the role of making available a new source of blood supply to the myocardium by redistribution, but in this respect the collateral vascular bed provoked by operation acts as an anastomotic bridge that may transport blood from the bed of one coronary artery to the bed of another coronary artery, where presumably blood flow may be deficient.

Of equal importance are the magnitude and speed with which these intercoronary communications developed following the procedure. In one instance, arterial sized intercommunicating channels had developed within 10 days.

Summary. 1. No interarterial intercoronary anastomoses between any branch of right and left coronary artery could be demonstrated at autopsy in normal dogs with a vinyl plastic injection mass. 2. Following surgical production of arteriovenous fistula (10-12 mm long) between main pulmonary artery and left atrium, interarterial intercoronary anastomoses between right and left coronary arteries were demonstrated at postmortem examination in 5 of 7 dogs examined at intervals of 2 days to 14 weeks following surgery. These anastomotic communications were arterial in size.

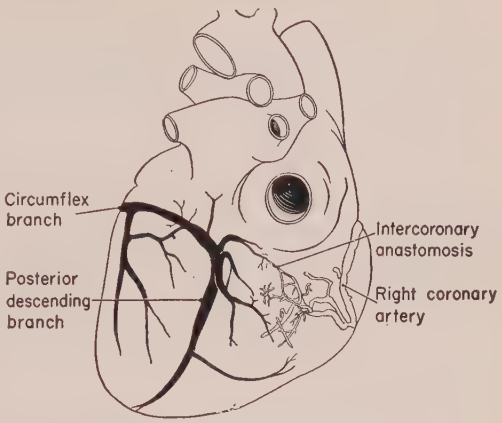

FIG. 1. Tracing of color photo of vinyl injection-corrosion cast to demonstrate intercoronary anastomosis between branch of left circumflex artery and right coronary artery 10 days following surgical production of a fistula between main pulmonary artery and left atrium. Outline of posterior surface of heart has been drawn in.

1. Van Slyke, D. D., Neill, J. M., *J. Biol. Chem.,* 1924, v61, 523.

2. Day, S. B., *J. Surgery,* 1957, v41, 220.

3. Zoll, P. M., Wessler, S., Schlesinger, M. J., *Circulation,* 1951, v4, 797.

4. Eckstein, R. W., *Circulation Research,* 1955, v3, 306.

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Paper Electrophoresis of Human Synovial Fluid.*† (24108)

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Paper electrophoresis of human synovial fluid was performed to determine electrophoretic distribution§ of protein fractions and to separate hyaluronate from proteins. Studies were done on synovial fluid obtained from normal subjects and from a few patients with osteoarthritis.

Methods. Synovial fluid was obtained from knee joints of post-mortem subjects(1) and from patients with osteoarthritis. One part (by weight) of sodium diethyl barbiturate buffer (pH 8.6, $\Gamma/2 = .075$) was mixed with 3 parts (by weight) of synovial fluid. This buffer contained 75 turbidity-reducing units (TRU) of testicular hyaluronidase (Wyeth)/0.1 g. Usually 0.1 g of buffer (containing 75 TRU of hyaluronidase) and 0.3 g of synovial fluid were used. This mixture was then incubated 4 hours at 37°C. Paper electrophoresis was performed in Spinco Model R apparatus with eight 3 cm Whatman 3MM papers and sodium diethyl barbiturate buffer (pH 8.6, $\Gamma/2 = .075$) at a voltage gradient of 3 volts/cm for 16 hours.

Standards. The hyaluronate standard was a sample of partially purified hyaluronate prepared from synovial fluid from a subject with osteoarthritis, and contained 23% hexosamine and 7% nitrogen. The normal serum standard was prepared from pooled normal sera. *Stains.* a) Bromphenol blue. An amount of synovial fluid or serum containing approximately 350 γ of protein was applied. The strips were stained with bromphenol blue as described by Block, Durrum, and Zweig(2), using staining time of 6 hours. Spinco Analytrol was used to determine amount of dye taken up by each protein fraction. b) Periodic acid Schiff (PAS). Synovial fluid or serum containing approximately 1200 γ of protein was applied. Staining was performed according to Köiw and Grönwall(3). c) Mucicarmine and toluidine blue. Synovial fluid containing 30 γ of hyaluronate hexosamine was applied. The staining of hyaluronate with mucicarmine and toluidine blue has been described elsewhere(4). Prior to staining with toluidine blue, the strips were heat-fixed at 100°C for 30 minutes and were not fixed in alcohol-formaldehyde solution as previously reported. *Analytical methods.* Protein concentration of synovial fluid was determined by biuret method(5) using normal serum as standard. The protein nitrogen of serum was determined by micro-Kjeldahl procedure(6). Hyaluronate was measured as hexosamine(1).

Results. 1) *Normal synovial fluid.* After digestion with hyaluronidase the migration by paper electrophoresis of proteins in normal synovial fluid was identical to migration of protein fractions of serum. Percentage of albumin was higher, and percentage of $alpha_2$ and of gamma globulins was lower in normal synovial fluid than in normal serum (Table I).

$Alpha_2$ globulin zone of normal synovial fluid stained much less intensely with PAS than the $alpha_1$ globulin zone. This is a reversal of the staining pattern of alpha globulins of normal serum with PAS(7) and may be due in part to decreased concentration of $alpha_2$ globulins in normal synovial fluid.

Hyaluronate of hyaluronidase-treated normal synovial fluid migrated faster than albumin, had a mobility identical to the similarly-treated hyaluronate standard, stained metachromatically with toluidine blue (Fig. 1), did not stain with bromphenol blue, was PAS

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§ By electrophoretic distribution of protein fractions of synovial fluid is meant amount of protein in each fraction separated by paper electrophoresis expressed as % of total protein of fluid.

TABLE I. Electrophoretic Distribution of Proteins of Normal and Osteoarthritic Synovial Fluid.

	Protein conc., g/100 g	Distribution of proteins				
		Albumin	Globulins			
			Alpha$_1$	Alpha$_2$	Beta	Gamma
		%				
Pooled normal sera	6.8	52	5	11	13	19
Normal synovial fluid						
Age, 1 yr		68	6	6	12	8
2		71	5	4	11	9
23	2.7	81	3	3	7	6
25		70	3	3	14	10
29	1.6	80	3	3	6	8
29		79	5	2	7	7
42	2.5	67	7	5	10	11
57	2.6	76	4	3	10	7
58	1.8	69	4	5	11	11
63	2.5	74	4	3	8	11
71		68	5	4	11	12
76	1.2	65	6	5	13	11
Osteoarthritic synovial fluid						
54		53	6	6	15	20
57	3.5	60	4	3	13	20
59	2.7	56	8	7	12	17
67	2.4	55	6	6	17	16
69	2.8	58	9	8	12	23

negative, and stained with mucicarmine.

2) *Osteoarthritic synovial fluid.*‖ After digestion with hyaluronidase, migration by paper electrophoresis of proteins and hyaluronate of osteoarthritic synovial fluid was identical to the migration of similar components of normal fluid. Percentage of albumin was lower and percentage of gamma globulins was higher in osteoarthritic fluid than in normal fluid (Table I). Percentage of albumin was slightly higher and percentage of alpha$_2$ globulins lower than in normal serum (Table I). Electrophoretic distribution of proteins in osteoarthritic serum was similar to normal serum.

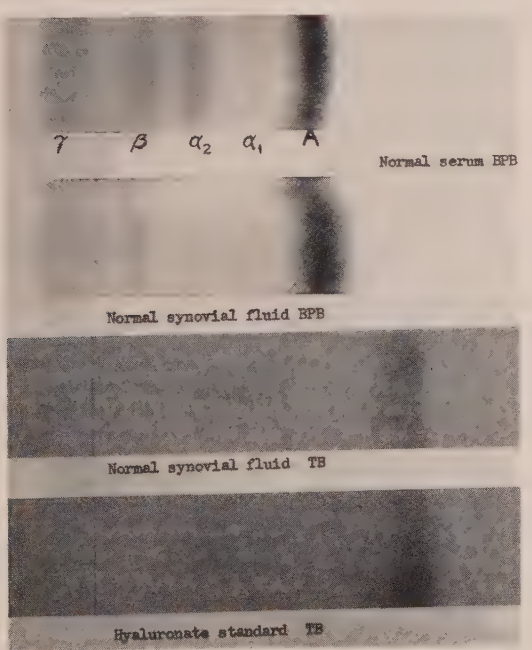

FIG. 1. Paper electrophoresis of hyaluronidase-treated normal synovial fluid, hyaluronidase-treated hyaluronate standard, and normal serum stained with bromphenol blue (BPB) and toluidine blue (TB).

Staining reactions of components of osteoarthritic fluid with bromphenol blue, PAS, mucicarmine, and toluidine blue were identical to those of normal fluid.

Discussion. The use of paper electrophoresis to determine distribution of proteins of normal synovial fluid has overcome difficulties encountered by workers using free electrophoresis. In a recent study(8) of normal synovial fluid by free electrophoresis, the concentration of globulins was too low to permit electrophoretic distribution of proteins in

‖ Synovial fluid obtained from osteoarthritic joints will be briefly termed osteoarthritic synovial fluid.

these fractions to be determined. The small volume of synovial fluid in normal joints has led some investigators(9) to use fluid from traumatized joints as a basis with which to compare changes in electrophoretic distribution of synovial fluid proteins in diseases of the joints, but an increased volume of fluid and a higher concentration of protein set traumatic synovial fluid apart from normal fluid. The technic of paper electrophoresis reported here has permitted analysis of very small amounts of synovial fluid from normal subjects ranging in age from 1 to 76 years and has revealed marked differences in electrophoretic distribution of protein fractions of normal synovial fluid and normal serum. Differences in distribution of synovial fluid proteins with age were not found.

Electrophoretic distribution of proteins in synovial fluid of a few patients with osteoarthritis was determined. Distribution of protein in these fluids differed from that found in synovial fluid obtained from normal subjects of a similar or older age than patients with osteoarthritis. This suggests that the underlying process in osteoarthritis is not simply one of "accelerated normal aging."

Permeability(10) of normal synovialis may determine which plasma proteins gain access to the synovial fluid, but factors other than permeability must operate to keep the albumin concentration (% albumin x protein concentration—Table I) of normal fluid at a level one half that of plasma.

Staining reactions of hyaluronate separated from proteins of hyaluronidase-treated synovial fluid by paper electrophoresis were of interest. Failure of bromphenol blue to stain hyaluronate indicated that the protein content of hyaluronate was low. Hyaluronate did not give a positive PAS reaction. This finding agrees with reports(11,12) that hyaluronate fails to react with PAS when it is free of protein-bound components which stain with PAS. Hyaluronate stained metachromatically with toluidine blue although partially depolymerized by hyaluronidase. Depolymerization of synovial fluid hyaluronate was indicated by the fact that the relative viscosity of normal synovial fluid, measured in an Ostwald viscometer at 37°C, fell from a value exceeding 500 to 1.5 within 4 hours after the addition of testicular hyaluronidase in the barbital buffer (pH 8.6). Since the pH of the synovial fluid was not lowered to 5 prior to addition of hyaluronidase, depolymerization of hyaluronate was not sufficient to render it dialyzable(1). Persistence of metachromasia of depolymerized hyaluronate has not been previously noted, and is of interest since depolymerization has been thought to destroy the chromatropic property of hyaluronate(13).

Summary. After treatment of human synovial fluid with hyaluronidase, the hyaluronate and proteins can be separated by paper electrophoresis. The electrophoretic distribution of the protein fractions of normal synovial fluid differs from that of the protein fractions of both normal serum and osteoarthritic synovial fluid.

1. Hamerman, D., Schuster, H., *J. Clin. Invest.*, 1958, v37, 152.

2. Block, R. J., Durrum, E. L., Zweig, G., *Paper Chromatography and Paper Electrophoresis,* Paper Part II, Academic Press, 1955.

3. Köiw, E., Grönwall, A., *Scand. J. Clin. Lab. Invest.*, 1952, v4, 244.

4. Hamerman, D., *Science,* 1955, v122, 924.

5. Wiechselbaum, T. E., *Am. J. Clin. Path., Tech. Sect.*, 1946, v16, 40.

6. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry,* Charles C. Thomas, Springfield, 1948.

7. Björnesjo, K. B., *Scand. J. Clin. Lab. Invest.*, 1955, v7, 153.

8. Platt, D., Pigman, W., Holley, H., Patton, F., *Arch. Biochem. Biophys.*, 1956, v64, 152.

9. Perlman, G. E., Ropes, M. W., Kaufman, D., Bauer, W., *J. Clin. Invest.*, 1954, v33, 319.

10. Ropes, M. W., Bauer, W., *Synovial Fluid Changes in Joint Disease,* Harvard Univ. Press, Cambridge, Mass., 1953.

11. Davies, D. V., *Stain Tech.*, 1952, v27, 65.

12. Hooghwinkel, G. J. M., Smitz, G., *J. Histochem. Cytochem.*, 1957, v5, 120.

13. Discussion in First Conf. on Connective Tissues, Josiah Macy Jr. Fn., N. Y., 1950, 93-95.

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Propagation of Measles Virus in Suckling Mice.* (24109)

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Because of the interest in immunological similarity between measles and distemper viruses(1), an attempt to adapt measles virus to mice was undertaken to establish a common animal host. The following experiments indicate that measles virus may be propagated in the central nervous system of suckling mice. Earlier reports on successful transmission of measles virus in mice have been described, but further attempts have failed to produce convincing evidence of its propagation(2). Since the report by Enders and Peebles(3) on successful isolation of measles virus in cultures of human and monkey renal epithelial cells, other cell cultures supported continuous multiplication of the virus(1,4,5,6). Propagation of measles virus (Edmonston strain) in tissue culture has made possible its adaptation to chick embryo(7), chick cells in culture(8), and, as is here reported, its multiplication in the central nervous system of suckling mice.

Materials and methods. Virus. The Edmonston strain(3) of measles virus was propagated in HeLa cell culture(1) and supernatant fluids from infected cultures were used as inoculum for initial passage. *Mice.* Laboratory-bred litters of Swiss albino mice, 1-2 day old, were inoculated intracerebrally in right hemisphere with 0.01 ml of inoculum. *Passages and titrations.* A 10% suspension of infected brain was prepared in brain heart infusion broth. All suspensions were centrifuged at 3,000 rpm for 15 minutes and contained 500 units of penicillin and 200 μg of streptomycin/1 ml of inoculum. For titrations, 10 fold serial dilutions were prepared in brain heart infusion broth. *Tissue culture.* HeLa cell cultures were grown and maintained in medium containing 60% yeast extract medium(9), 20% Scherer's maintenance solution (10), 10% normal calf serum and 10% brain heart infusion broth. The medium was changed every 3-4 days. *Serum neutralization tests.* Mixtures of diluted serum and 20% infected mouse brain, in equal volumes, were held at room temperature for ½ hour at 4°C for additional ½ hour before inoculation of HeLa cell cultures. All serum samples were inactivated at 56°C for ½ hour before mixing with the virus. A standard inoculum of 0.2 ml was added to HeLa cell culture tubes and observed daily for 21 days. The technic for neutralization studies in suckling mice was similar to above procedure. A 20% brain suspension was mixed with dilutions of serum, and incubated similarly before injecting 0.01 ml intracerebrally into suckling mice. Death of animals was recorded for 21 days and neutralization was determined by survival of animals. *Normal and immune sera.* Neutralization tests were carried out with dilutions of acute and convalescent serum from patients with typical measles. Tests were also carried out with sera from ferrets as described earlier(1). Normal ferret serum was obtained from animals prior to immunization and the specific immune serum was obtained 1 month after final injection of tissue culture measles virus.

Results. Passage of measles virus in suckling mice. Supernatant fluid from measles infected HeLa cultures was inoculated intracerebrally into suckling mice of 2 litters. Mice of one litter were sacrificed on 4th and 7th day after inoculation and no virus was detected when brain material was assayed in HeLa cultures. The second litter was observed for signs of illness and 10 days after inoculation, one mouse showed definite signs of irritability. This mouse was sacrificed and a 10% suspension of brain tissue was prepared. The material was inoculated into HeLa cultures in 0.1 ml volumes for detection of virus and also passed serially in suckling mice. The earliest manifestation of illness consisted of running and jumping in the cages.

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TABLE I. Intracerebral Passage of Measles Virus in Suckling Mice.

Passage No.	Ration of infection	Incubation (days)	Mouse titer, log LD_{50} /.01 ml	HeLa titer, log TCD_{50} /.1 ml
1	1/ 7*	10		1.7
2	7/ 7	6		3.2
3	15/20	8		2.5
4	16/20	9–11	1.2	2.5
5	9/16	10		1.5
9	7/17	9–12	1.5	
11	7/13	8– 9	1.4	2.5
12	13/19	8–10	1.3	2.5
17	8/14	7	1.4	2.0
22	11/12	9		3.2
24	11/12	7	2.0	

* Animals either dead of infection or sacrificed when moribund over total No. of animals inoc.

Subsequently, animals became apathetic, lying on their sides and moving very slowly when disturbed. Death generally occurred 2-3 days after initial signs of illness. All passage material when assayed in tissue culture produced a cytopathogenic effect similar to that associated with the original HeLa measles virus(1). Observations of passage study and results of virus titrations in HeLa cultures and suckling mice are summarized in Table I. The approximate LD_{50} titers in suckling mice were quite constant and ranged between $10^{1.2}$ and 10^{2}/0.01 ml for 24 mouse passages. The mouse propagated agent produced cytopathology in HeLa cell cultures and gave TCD_{50} titers between $10^{1.5}$ and $10^{3.2}$/0.1 ml. These results correlate well with the infective titers in suckling mice.

Preservation of mouse measles virus. Ten percent suspensions of brain material from 3rd and 7th passages were stored at –35°C for 7 and 2 months respectively. When 7th passage material was thawed and injected into suckling mice, the comparative infection rates of original and frozen material showed no significant difference. Virus was still viable after 7 months of storage, however, there was some loss of infectivity as shown by a 60% decrease in death rate.

Identification of suckling mouse propagated virus. Three principal methods were employed to identify mouse propagated virus as measles virus: 1) Serum neutralization tests in tissue culture, 2) Serum neutralization tests in suckling mice, and 3) Protection tests in suckling mice. Neutralization tests in tissue culture were performed with specific measles antisera prepared in ferrets and with human measles convalescent sera. For control purposes normal ferret sera (pre-immunization) and human measles acute sera were employed. Immune ferret sera and human convalescent sera completely inhibited the cytopathogenic effects of the virus, whereas, normal ferret sera and acute human sera showed no significant inhibitory effect. The results are summarized in Table II. Neutralization studies in suckling mice were carried out with virus obtained from 22nd mouse passages. These results correlated well with neutralization studies in tissue culture. Ferret antimeasles sera and human measles convalescent sera protected 50% of mice against lethal effects of the virus at 1/16 and 1/128 dilutions of the sera respectively, whereas, normal ferret sera and acute human sera showed no observable neutralizing effect. In protection tests of suckling mice, adult female mice were immunized by multiple injections of HeLa adapted measles virus. Pups born from these mice were protected from challenge inoculation of mouse propagated measles virus, whereas mice born from females immunized with control material or from untreated females succumbed to the challenge. These results are summarized in Table III.

Serial propagation of mouse measles virus in HeLa culture. After 4th mouse passage, serial transfer of mouse adapted measles virus in HeLa cell culture was difficult when supernatant fluids were employed; however, cytopathogenesis was readily induced when infected cells were used as inoculum. This phenomenon was also observed when suckling

TABLE II. Neutralization of Mouse Propagated Virus with Measles Antiserum in HeLa Culture.

Mouse passage	Serum samples	Neutralizing serum dilution
11	Human acute	1/8
	" convalescent	1/128
17	" acute	<1/4
	" convalescent	1/128
13	Normal ferret	<1/4
	Ferret antimeasles	1/32
17	Normal ferret	<1/4
	Ferret antimeasles	1/16

TABLE III. Protection Test in Suckling Mice.

Mouse measles employed	Suckling mice born from mothers Immunized with measles virus	Immunized with control material	Untreated
7th passage	0/ 8*		6/6
8th "	0/ 9	5/9	4/9
9th "	0/17	3/9	4/6

* No. dead/Total No. inoculated.

mice were inoculated with tissue culture material, as illustrated in the following experiment. Virus representing 20th mouse passage was back titrated in HeLa cell culture and produced characteristic cytopathogenesis. When cytopathogenicity had reached 4 plus after 9 days of incubation, culture fluids were removed, and centrifuged twice at 3,000 rpm for 15 minutes. The supernatant fluid when back titrated in mice did not exhibit any lethal effect, whereas the sediment consisting of cellular debris produced typical signs of infection and eventual death. Further passage studies showed that these deaths were due to the measles virus.

Discussion. After 24 serial passages of measles virus in suckling mice, an increase of virulence failed to manifest itself. Unlike many other viruses, neither the infective titer of mouse brain material nor mortality rate was appreciably altered. Multiplication of virus in the central nervous system was evidenced by presence of more virus in each passage than the amount present in the original inoculum. In experiments with dengue virus, up to 15 passages were required before uniform clinical signs of infection were evident (11). It is possible that additional mouse passages of the measles virus are necessary before lethal infection will occur uniformly in all mice. In back titration experiments, fluid phase from cultures inoculated with late passage materials did not contain infectious material either for HeLa cells or for suckling mice. Infections, however, were readily induced when cells were employed as inoculum. Failure of infectious agents to be liberated into supernatant fluid is peculiar since culture fluids from back titration of mouse adapted agent were obtained after 4 plus destruction of HeLa cells. In contrast, our unpublished experiments, which agree with data reported by Ruckle(6), show that tissue culture adapted measles virus can be detected in the fluid phase on the same day as the occurrence of first obvious cytopathic changes. The possibility of incomplete viruses being liberated into the supernatant fluid is being investigated.

Summary. HeLa adapted measles virus (Edmonston strain) was propagated in suckling mice through 24 serial intracerebral passages. The virus produced a lethal infection in most animals 7 to 13 days following inoculation. Identity of mouse propagated agent was established by serum neutralization tests in tissue culture and in suckling mice. Further identification was established by protection of suckling mice born of mothers immunized with tissue culture measles virus. Fluid phase from tissue cultures inoculated with late mouse passage materials did not contain infectious material either for HeLa cells or for suckling mice. Infections, however, were readily induced when cells were employed as inoculum.

1. Adams, J. M., Imagawa, D. T., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 240.

2. Taniguchi, T., Okuno, Y., Kamahora, J., Aoyama, A., Kato, S., *Med. J. Osaka Univ.,* 1954, v4, 381.

3. Enders, J. F., Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

4. Black, F. L., Reissig, M., Melnick, J. L., *ibid.,* 1956, v93, 107.

5. Dekking, F., McCarthy, K., *ibid.,* 1956, v93, 1.

6. Ruckle, G., *J. Immunol.,* 1957, v78, 330.

7. Milovanovic, M. V., Enders, J. F., Mitus, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 120.

8. Katz, S. L., Milovanovic, M. V., Enders, J. F., *ibid.,* 1958, v97, 23.

9. Robertson, H. E., Brunner, K. T., Syverton, J. T., *ibid.,* 1955, v88, 119.

10. Scherer, W. F., *Am. J. Path.,* 1953, v29, 113.

11. Sabin, A. B., Schlesinger, R. W., *Science,* 1945, v101, 640.

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Minimal Infectious Inoculum of *Histoplasma capsulatum* for Mouse and Chick Embryo. (24110)

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Until Emmons(1) first isolated *Histoplasma capsulatum* from the soil, little was known about its habitat and mode of distribution in nature. Following its isolation from the soil it was hypothecated that the fungus may be carried with dust and that it probably enters the host through the respiratory tract. Later studies by Ajello and Zeidberg(2) Grayston *et al.*(3), Ibach *et al.*(4) have substantiated this hypothesis especially with dust and debris associated with chicken houses, silos and storm cellars. Since the yeast phase has been shown by laboratory studies to be very sensitive to environmental conditions, the fungus is thought to exist in nature in the mycelial phase. Histoplasmosis has been produced in animals by inoculating with the yeast phase or with portions from the entire mycelial phase. Few qualitative or quantitative studies have been made to determine the actual constituents of the mycelial phase used as the inoculum. For these reasons it has been difficult to establish unequivocally the actual fragment portions of the fungus causing infection in laboratory animals. There are many predisposing factors which influence infectivity of the mycelial phase, one of which is the amount of the inoculum. The determination of the minimal inoculum to induce infection in animals has been adequately documented for only the macroconidium of the fungus(5,6). The regularity with which animals can be infected with fragments of the mycelial phase has not been reported. This investigation was undertaken to determine, if possible, the minimal inoculum necessary to induce infection and the regularity with which animals can be infected with this amount of inoculum. A single spore or fragment of the mycelial stage of the fungus was taken as the smallest practical amount of inoculum to use. On this basis animals were injected with single particles as follows: (1) non-germinated tuberculated macroconidia, (2) germinated tuberculated macroconidia, (3) non-germinated microconidia, (4) germinated microconidia, and in addition(5) a non-branching mycelial fragment.

Materials and methods. The strain of the organism used in the first series of experiments was a culture of the Ellis isolate of *H. capsulatum* which had been grown on Sabouraud's glucose agar and allowed to remain on the same medium for 3 months without transfer. In the second series of experiments, in which only chick embryos were inoculated, the Ellis isolate had been grown on corn grains and allowed to remain on the same grains for 3 years. Each of the 5 types of inocula from the mycelial phase was isolated using a Chambers micromanipulator. The mycelial phase of the fungus was suspended in a drop of Sabouraud's broth which contained 40 μg streptomycin and 20 units penicillin per ml. A moisture chamber as described by Richter (7), with addition of 2 small copper capillary tube holders, was used to prevent evaporation of the hanging drop. Each unit of inoculum was introduced into a sterile glass capillary tube which was placed aseptically inside the shaft of a one inch, 21 gauge hypodermic needle. A small ball of semi-firm rubber cement was placed in the needle shank to prevent leakage. The needle was attached to a Leur Loc syringe which contained 40 μg streptomycin and 20 units of penicillin per ml of sterile saline. Each unit of inoculum was observed microscopically when placed in the capillary tube just prior to inoculation. The unit was placed toward the center of the capillary tube to minimize possibility of loss prior to inoculation. After each inoculation the capillary tube was examined microscopically for the presence of the inoculum. Since the inoculum was never found in the capillary tube it was assumed that it had been transferred

to the host and that infection, if any, was the result of the inoculation. Each white Swiss mouse was inoculated intraperitoneally. The capillary tube containing the inoculum was always placed a small distance back of the point of the needle to prevent breakage or loss of the inoculum during the inoculation procedure. Mice dying within 72 hours after inoculation were considered as traumatic deaths and discarded. Any animal dying after the third day was autopsied and the liver, spleen and adrenals were removed and ground separately in a sterile mortar using sterile sand and saline. One ml of each tissue and extract was streaked separately onto one blood agar plate and on each of 2 Sabouraud's agar plates which contained 40 μg streptomycin, 20 units of penicillin, and 1.5 mg of actidione per ml of medium. The remainder of the mice were maintained for 8 weeks, then sacrificed and autopsied. The liver, spleen and adrenals of these mice were treated as above and cultured separately on Sabouraud's agar. The same procedure was followed for the control mice which had been inoculated with sterile saline which contained the antibiotics. The yolk sac of 5-day-old White Leghorn embryos was inoculated aseptically through a perforation in the shell over the air sac. The perforation was sealed with collodion. The eggs were incubated at 33°C in a Bower's humidaire incubator automatically set to rotate every 6 hours. The eggs were candled daily. The eggs containing dead embryos were discarded on the third day. Embryos which died after the third day were triturated and streaked on Sabouraud's and blood agar containing the above listed antibiotics. After 18 days incubation the living embryos were sacrificed, autopsied, and the liver, spleen, and adrenals were triturated and portions of each streaked on modified Sabouraud's agar. When these organs had not developed, the complete embryo was triturated and streaked. A series of embryos was inoculated with sterile saline and antibiotic solution. These embryos were cultured using the same procedure as above and were used as controls. Impression smears were made of various tissues of mice and chick embryos, including yolk sac. These smears were stained with Giemsa and observed under oil immersion for yeast-like type of cells of the fungus. Mouse and chick embryo tissues were incubated at room temperature for 6 weeks on the artificial media and examined periodically for the presence of the fungus.

Results. The percentage of white mice and chick embryos infected by single particles of inoculum from the 3 months' culture of *H. capsulatum* grown on Sabouraud's glucose agar is presented in Table I. The results indicate that infection in mice takes place following injection with a single spore of each type used but not with the non-branching mycelial fragments. Non-germinated tuberculated macroconidia proved the most and non-germinated microconidia the least infective of the spores. In chick embryos the germinated tuberculated macroconidia were 3.6 times more infective than were non-germinated macroconidia, just the reverse of that found in white Swiss mouse.

Results from chick embryos inoculated with single units of inoculum from a 3-year-old culture of fungus grown on corn grains are shown in Table II. Each type of inoculum was capable of causing experimental histoplasmosis.

TABLE I. Percentage of White Mice and Chick Embryos Infected by Single Particle Inoculum of 3-Months-Old Culture of the Ellis Isolate of *Histoplasma capsulatum*.

Units inoculated	White mouse		Chick embryo	
	Infective ratio	% infected	Infective ratio	% infected
Non-germinated tuberculated macroconidia	4/48	8.2	0/40	.0
Non-germinated microconidia	1/47	2.1	3/42	7.1
Germinated tuberculated macroconidia	3/48	6.2	7/51	13.7
Germinated microconidia	1/19	5.3	3/51	5.9
Non-branching mycelial fragments	0/20	.0	4/28	14.3
Total	9/182	5.0	17/212	8.0

x/n = No. of animals infected/No. of animals inoculated.

TABLE II. Percentage of Chick Embryos Infected by Single Particle Inoculum of 3-Year-Old Culture of Ellis Isolate of *Histoplasma capsulatum.*

Units inoculated	Infective ratio	% infected
Non-germinated tuberculated macroconidia	4/73	5.5
Non-germinating microconidia	6/69	8.7
Germinating tuberculated macroconidia	4/65	6.2
Germinating microconidia	6/71	8.5
Non-branching mycelial fragments	9/81	11.1
Total	29/359	8.1

x/n = No. of chick embryos infected/No. of chick embryos inoculated.

The non-germinated tuberculated macroconidia were the least infective of the inocula used. Microconidia proved to be more infective than macroconidia and there was no significant difference between germinated and non-germinated microconidia. In these, as in earlier studies, the mycelial fragments were the most infective in White Leghorn chick embryos.

In addition to white Swiss mouse and leghorn embryos, 2 other series of experiments were completed using a hybrid chick embryo and a C57 strain of black mice. In one instance hybrid fertile eggs were inadvertently supplied and these were inoculated prior to knowledge of the error. This hybrid was a cross between White Leghorn cockerel and New Hampshire Red hen. Although the same technics were followed in inoculating and culturing the hybrid embryos, striking differences in infectivity of the particles occurred. Of the 129 hybrid embryos living 72 hours after inoculation only 4 developed an infection. The mycelial fragment was the only infective particle in that 4 of 28 embryos yielded the fungus on culture. No infection occurred in the C57 strain of black mice inoculated with germinated tuberculated macroconidia. Nineteen of these animals received the inoculum intraperitoneally and 15 by inhalation route. Black mice were inoculated since it has been stated that pigmented animals are more susceptible to certain pathogenic fungi than are the white strains (Personal communication from N. F. Conant).

Tissue impression smears from autopsied animals were made with the hope of determining the fate of the inoculated particles. In many of the stained smears, especially the yolk sac preparations, yeast-like cells were observed using the oil immersion lens. These findings indicate that the mycelial phase does not lay dormant in the animals only to germinate when tissues were cultured on artificial media. Similar observations were made in our earlier study(6) in which yeast-like cells were photographed in tissue smears and sections.

Non-germinated tuberculated macroconidia were individually placed on artificial media to check rate of germination. Quadrant plates were used and presence of spores was ascertained by microscopical observation. More than 150 single macroconidia were studied and in no case did one of these spores germinate. These results indicate that the germination rate of *H. capsulatum* single spores is extremely low.

Discussion. Few studies have been made using a single spore or a fragment of the hypha of *H. capsulatum* to infect animals. Ajello and Runyon(5) reported implantation of single tuberculated macroconidia on agar plugs into the peritoneal cavity of mice. This method proved highly successful in that nearly 100% of the mice were infected. No statement was made by authors as to whether macroconidia were germinated or non-germinated. In another study Larsh *et al.*(6) reported successful infection of chick embryos with a small number of spores as determined by hemacytometer and viability counts. These infection rates were correlated with viability of the inoculum on potato dextrose agar plates. An infectivity rate as high as 41% was reported when 2 spores were used. These investigators made no attempt to produce infection in animals by injection of a single tuberculated macroconidium.

The results reported herein give the first experimental evidence of infectivity of *H. capsulatum* single units of inoculum other than tuberculated macroconidia. To eliminate the inherent criticism of establishing unequivocally the size of the inoculum for these infectivity studies, a Chambers micromanipulator

was used. It was feasible to isolate single fungal elements with this instrument and to place them in sterile glass capillary tubes for inoculation. Presence of the inoculum in the tube was verified in each instance by microscopical observations. In addition, post-inoculation, the capillary was examined microscopically to ascertain presence or absence of the inoculum. This procedure proved tedious, time-consuming, and painstaking one but resulted in establishing the fact that a single spore or fragment of hypha would bring about infection.

Although inoculation of mice and chick embryos was done using aseptic technic, to aid in preventing bacterial contamination of the inoculum, streptomycin and penicillin were included in the collecting and inoculating fluids. This procedure was followed since the content, especially of yolk sacs, was a good medium for growth of contaminating organisms. In the control series it was learned that these antibiotics did not have any apparent deleterious effect upon embryos, white mouse, or growth of fungus. No deaths of animals could be attributed to contaminating organisms. The inoculation procedure proved highly successful and the method did not offer any physical protection to the infectious agents. The use of extraneous material in the inoculum may in part account for the extremely high infectivity rates reported by other investigators.

The macroconidia were more infective than microconidia in White Swiss mice. The differences in infectivity rate of the 2 types of macroconidia were not too significant but the germinated microconidia were 2.5 times more infective than were non-germinated microconidia. In all mouse series the number of infected animals was small and no trend of enhanced invasiveness of any unit was apparent.

It is of particular interest that in the 2 series of chick embryos, inocula of different ages were used, nevertheless, total infectivity in each instance was strikingly similar. The sources of fertile eggs were different but were all from White Leghorn hens. In both chick embryo series the non-branching mycelial fragments produced highest infectivity rate. In the case of 3-year-old corn grain culture this finding would seem to be of significance and may indicate what happens to the fungus in nature.

Summary. (1) Individual infectious particles were isolated from the mycelial phase of *H. capsulatum* using Chambers micromanipulator. The particles consisted of germinated and non-germinated tuberculated macroconidia, germinated and non-germinated microconidia and non-branching mycelial fragments. Each particle was placed into a sterile capillary tube inserted into a 21 gauge sterile inoculating needle. Aseptic technics were followed, in addition, streptomycin and penicillin were added to the collecting and inoculating fluids. Microscopical examination of each capillary tube was made prior to and immediately after inoculation procedure to ascertain the disposition of the particle. Fertile Leghorn eggs and the white Swiss mouse were the experimental animals. (2) Infection resulted from all particles with one exception, mycelial fragments, in the mouse. The total percentage of infectivity in the 182 inoculated mice was 5%. Of the 571 inoculated chick embryos approximately 8% became infected. The most infectious particle was the mycelial fragment. (3) Inoculation of single particles from the mycelial phase of *H. capsulatum* proved that the minimal infection inoculum can be units other than the macroconidium. In each instance the infectivity rate was relatively small but no physical protection was given the inoculated particle.

1. Emmons, C. W., *Pub. Health Rep.,* 1949, v64, 892.

2. Ajello, L., Zeidberg, L. D., *Science,* 1951, v113, 662.

3. Grayston, J. T., Loosli, C. G., Alexander, E. R., *ibid.,* 1951, v114, 323.

4. Ibach, M. J., Larsh, H. W., Furcolow, M. L., Proc. Soc. Exp. Biol. and Med., 1954, v85, 72.

5. Ajello, L., Runyon, L. G., *Pub. Health Monogr.,* 1952, v39, 93.

6. Larsh, H. W., Hinton, A., Cozad, G. C., *ibid.,* 1952, v39, 110.

7. Richter, K. M., *Science,* 1948, v108, 192.

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Established Kidney Cell Lines of Normal Adult Bovine and Ovine Origin.* (24111)

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The literature contains few references to established cell lines derived from normal adult domestic animal tissues. Drew(1) reported an established cell (epithelioid) obtained from normal kidney of adult female white rabbit, and Brion and Gruest(2) reported isolation of epithelial cells from calf kidney, which could be maintained *in vitro*. In addition to these, Haff and Swim(3) reported cell lines from normal adult rabbit muscle and testicle, and a normal adult rabbit kidney cell line, all of which were fibroblastic. Westwood *et al.*(4) reported cell lines from embryo rabbit kidney, Parker *et al.*(5) from rabbit kidney and embryo chick. Stice (unpublished) developed an established swine kidney cell which has been used extensively in this laboratory. No further reports have been found in the literature concerning cell lines obtained from normal adult domestic animal tissues.

It is the purpose of this paper to describe 2 newly established cell lines, one obtained from a normal adult bovine kidney (MDBK), and the other from a normal adult ovine kidney (MDOK).

Materials and methods. Nutrient medium (LA) consists of modified Earle's salt solution and lactalbumin hydrolysate, fortified with lamb serum, as follows: Sol. 1, modified Earle's NaCl 70 g, KCl 4 g, $CaCl_2$ 2 g, $MgSO_4 \cdot 7H_2O$ 2 g, NaH_2PO_4 1.4 g, dextrose 10 g, penicillin (potassium G) 2 g, streptomycin sulfate 1 g, polymixin B 1 g, phenol red (0.2% stock sol.) 10 ml, distilled water to 1000 ml. Sterilize by filtration and dispense in 100 ml amounts. Sol. 2: lactalbumin enzymatic hydrolysate 50 g, distilled water 1000 ml. Dispense this suspension in 100 ml amounts and sterilize by autoclaving at 10 lbs for 10 min. Sol. 3: sodium bicarbonate 75 g, distilled water 1000 ml. Sterilize by autoclaving at 15 lb for 15 min. The complete nutrient medium is obtained as follows: Sol. 1, 100 ml; Sol. 2, 100 ml; Sol. 3, 6-12 ml; lamb serum (sterile) 100 ml; distilled water (sterile) 800 ml.

Preparation of glassware and rubber stoppers. The method described by Madin *et al.* (6) was used throughout. *Preparation of primary tissue cultures.* Original cultures, from which all cell lines were derived, were prepared according to the methods of Madin *et al.*(6). *Cell resuspension fluid.* The saline (ATV) is a modification (A. J. Hackett, unpublished), of that described by Marcus *et al.* (7) and contains versene and trypsin as follows: (10X conc.) : NaCl 80 g, KCl 4 g, dextrose 10 g, $NaHCO_3$ 5.8 g, trypsin† 5 g, versene‡ 2 g, penicillin, potassium G 2 g, streptomycin sulfate 1 g, polymixin B 1 g, phenol red 0.2% stock sol. 10 g, distilled water to 1000 ml. This solution is sterilized by filtration and dispensed in 100 ml amounts. All solutions, unless otherwise stated, are stored at −17°F.

Preparation of cell lines. A vigorous culture of the primary cell growth is selected as cell source for passage. Usually 3 Blake bottles are used, one to serve as source of cells for continuous culture studies and 2 as a reserve cell source. The nutrient fluid is discarded, and the culture is quickly rinsed once with ATV, following which 25 ml of fresh ATV is added to aid in cell liberation. The bottle is placed in incubator at 37°C for approximately 15 min. The suspended cells are centrifuged 5 min. at 1000 rpm, resuspended in 10 ml of fresh nutrient media, and vigor-

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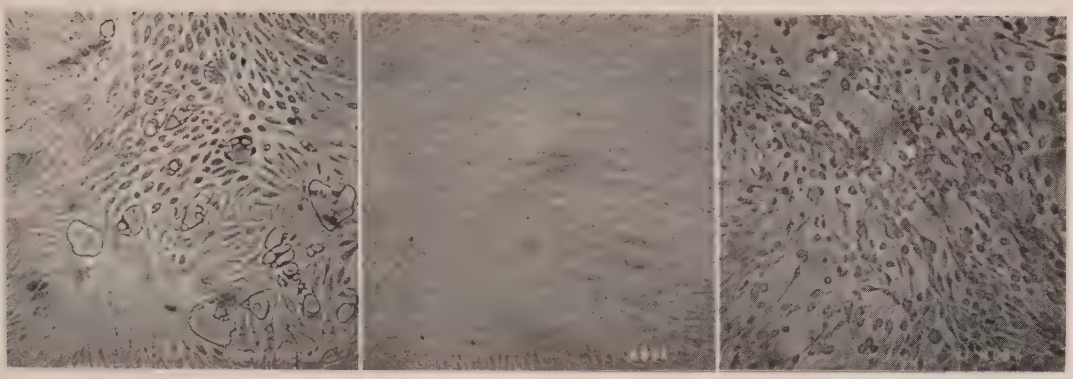
† Trypsin-1:250 Difco Laboratories, Detroit.

‡ Versene-disodium ethylenediamine tetraacetate, Fisher Scientific Co., Fairhaven, N. Y.

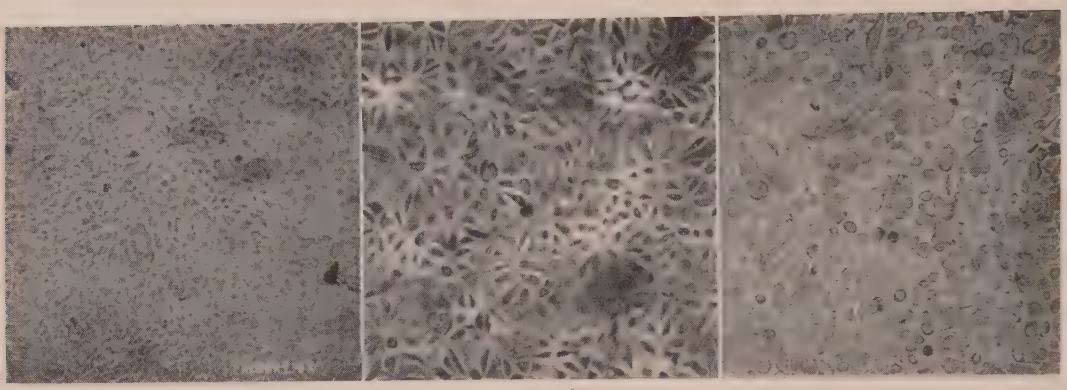


a b c

FIG. 1. a. Primary bovine kidney cells, 100×. b. Established bovine kidney cells in 32nd subculture (MDBK), 100×. c. Vesicular stomatitis virus-infected MDBK cells, 100×.

ously aspirated in a pipette until a homogeneous suspension is obtained. A cell count of this suspension is made with the aid of a hemocytometer and subsequent dilutions are made in nutrient media to yield a final concentration of approximately 5 x 10^4 cells/ml. Approximately 65 ml of this mixture is then added to each Blake bottle used to constitute the new passage. The cultures are incubated at 37°C for 7 days at which time they are satisfactory for repassaging. To obtain maximum growth, the nutrient fluid is changed on the 3rd or 4th day.

History of cell lines. The MDBK cell was carried through the first 16 serial passages on lactalbumin-Tris medium described by Madin *et al.*(6), with 30% lamb serum. The 17th through 22nd passage was made using the same medium with 10% lamb serum. All subsequent passages were made with LA medium described earlier. This particular culture exhibited no striking changes in morphology at any single passage. However, starting from a number of morphologically different cell types, a fibroblast-like cell became predominant at the 9th or 10th passage, and remained stable without any significant morphological changes throughout succeeding passages. Cultures inoculated with 5 x 10^4 cells/ml yield 10^7 cells/ml within a 7-day period at current passage level. The appearance of cells at 1st and 32nd passages is shown in Fig. 1a and 1b. The MDOK cell was serially passaged 8 times in the medium described by Madin *et al.*(6) with 30% lamb serum. 10% lamb serum was used for the 10th passage, and all subsequent passages were made with LA medium containing 10% lamb serum. The MDOK cell in contrast to MDBK began exhibiting increasing numbers of fibroblast-like cells from 4th passage on, and began to decrease in growth rate. Despite appearance of fibroblast-like cells the culture remained mixed fibroblast-epithelial until 12th passage. During the first 12 passages the cells had been transferred every 7 to 14 days. The 12th passage was distributed in several 3 oz. prescription bottles because of a decrease in growth rate and loss of transferable cell population. Attempts to passage the combined cells of 2 or 3 prescription bottles grown for 14, 28, and 42 days, respectively, failed. Sixty-day-old cultures, however, were capable of serial passage. It should be emphasized that while the nutrient fluid was changed approximately twice a week, only the 60-day-old cultures were capable of being subcultured continuously. At the end of the 60-day period epithelial-like cells predominated, eliminating the fibroblast-like cell which had previously been present. This epithelial-like cell formed the basis for the 13th passage, gradually increasing in growth rate over the next 3 passages, until by the 16th passage the current cell appeared firmly established. All succeeding passages have been marked by a uniform growth rate similar to the MDBK cell. The current MDOK cell is morphologically dissimilar both in shape and size to the culture isolated directly from lamb kidney (Fig. 2a and 2b). Apparently a



a b c

FIG. 2. a. Primary ovine kidney cells, 100×. b. Established ovine kidney cells in 30th subculture (MDOK), 100×. c. Vesicular stomatitis virus-infected MDOK cells, 100×.

marked change in cell morphology occurred primarily during the 12th passage.

Results. The current cell lines have been serially passaged 50 and 40 times for the MDBK and MDOK, respectively. Extensive investigations to determine the "type" of cell proliferating in either cell line have not been carried out. The MDBK cell appears somewhat spindle shaped and may be fibroblastic in nature, while the MDOK cell appears more epithelial-like.

Both cells support the *in vitro* growth of vesicular stomatitis virus types Indiana and New Jersey (Fig. 1c and 1b) and infectious bovine rhinotracheitis virus. McKercher (unpublished) states that the MDOK cell will support the growth of the virus of sheep bluetongue. Warren and Cutchins(8) examined 23 different viruses for their ability to multiply in embryonic bovine cells and found 9 which were able to propagate. It is suggested that both cells may be of particular aid to those interested in general host-parasite relationships.

Summary. Two newly established cell strains capable of continuous cultivation, one derived from adult bovine kidney (MDBK) and one from adult ovine kidney (MDOK), are discussed. It is suggested that the strains may be useful for those interested in veterinary virology in particular and virus-host relationships in general.

1. Drew, R. M., *Science,* 1957, v126, 747.

2. Brion, G., Gruest, J., *Ann. Inst. Past.,* 1957, v92, 426.

3. Haff, R. F., Swim, H. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 200; 1957, v94, 779.

4. Westwood, J. C. N., *et al., Brit. J. Exp. Path.,* 1957, v38, 138.

5. Parker, R. C., *et al., Spec. Publ. N. Y. Acad. Sci.,* 1957, v5, 303.

6. Madin, S. H., Andriese, P. C., Darby, N. B., *Am. J. Vet. Res.,* 1957, v18, 932.

7. Marcus, P. I., Cieciura, S. J., Puck, T. T., *J. Exp. Med.,* 1956, v104, 615.

8. Warren, J., Cutchins, E. C., *Virology,* 1957, v4, 297.

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Fate of Bacteriophage Particles Introduced into Mice by Various Routes.* (24112)

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Bacterial viruses inoculated into an animal should, because of their size and biological composition, act in many ways as a physical simulant for animal viruses. Because of their inability to specifically adsorb and multiply in sensitive animal tissues and the inherent simplicity of quantitation, bacteriophage studies were undertaken to ascertain the effect of some of the anatomical and physiological barriers of the animal body to large doses of bacteriophage particles, to explain the occurrences that follow introduction of large numbers of virus particles into host animals. Nungester and Watrous(1) have shown that 2 hours after intravenous inoculation of staphylococcus phage into rats the spleens and livers contained greater amounts of viable phage particles than did the blood. Appelmans(2) showed that bacteriophage particles inoculated into guinea pigs were rapidly eliminated through urine and feces but bacteriophage preparations administered orally did not pass into the blood stream. This report deals with the persistence and localization as well as the translocation of bacteriophage particles introduced into mice.

Method. The phage strain was derived from *Bacillus megatherium* 899a (lysogenic), and is the clear plaque mutant C strain as described by Gratia(3). The sensitive strain *Bacillus megatherium* KM was utilized throughout this study for production of phage stock and assaying procedures. The medium for stock phage production and all assay procedures consisted of 2% Bacto-peptone in both broth and agar medium. Phage determinations were carried out by the pour plate method as described by Adams(4). Two-tenths ml of phage suspension containing 2 x 10^{10} particles/ml, was injected intraperitoneally into white Swiss mice. At indicated intervals, mice were etherized and blood samples taken by the cul-de-sac method. The animals were then immediately sacrificed and brains, livers, spleens, kidneys and intestinal tracts (large intestines and caecum) were isolated in sterile petri plates. The individual organs were homogenized in sterile containers utilizing a Virtis tissue homogenizer. After homogenizing, the tissue suspension was placed into sterile test tubes and the homogenizer cup washed with a volume of sterile distilled water to bring total volume to 2 ml. The combined suspension was centrifuged and the supernatant assayed for phage content. Blood samples taken by the cul-de-sac method were assayed directly. In experiments concerned with oral administration of phage suspensions, introduction was achieved by feeding one drop from an 18 gauge needle from which the bevel had been ground and also by the administration of 0.1 ml by gastric lavage. Care was taken to prevent any laceration to nasopharynx and gastric mucosa by utilizing polyethylene tubing which had been flamed to remove any rough edges. Mice were lightly anesthetized with sodium pentathol before insertion of tube. Titer of phage suspensions in the feeding experiments was 1 x 10^{10}/ml. Blood samples were taken by cardiac punctures with heparinized syringes. In experiments when urine and feces were assayed for phage particles the following technics were used. Two drops of urine were suspended in 1 ml of sterile distilled water and assayed, and a fecal pellet was expressed from each animal, placed into 2 ml of sterile distilled water, emulsified, and the centrifugate assayed.

Results. Fig. 1 illustrates number of active phage particles recoverable from lung, kidney, brain and intestines at different time intervals after intraperitoneal inoculation of a phage suspension. Fig. 2 gives the same in-

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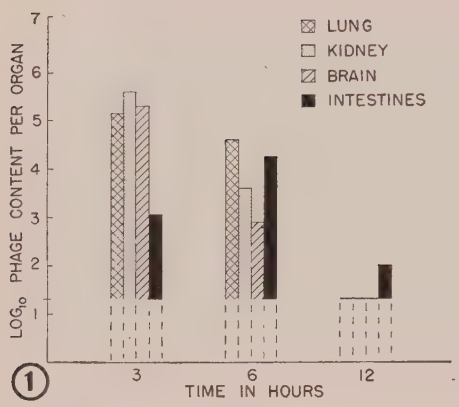

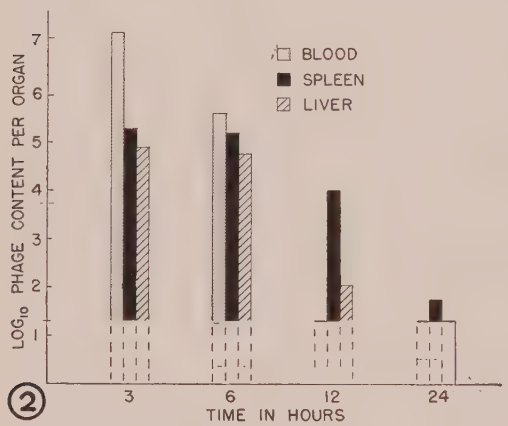

FIG. 1. No. of viable *B. megatherium* bacteriophage recoverable from lung, kidney, brain and intestine of mice calculated for content per total organ.

FIG. 2. No. of viable *B. megatherium* bacteriophage recoverable from blood, spleen and liver of mice calculated for content per total organ.

formation for blood, spleen and liver. For purposes of convenience and comparison all calculations have been made for active phage content/total organ. Since total phage inoculated was 4×10^9 particles, it can be seen that by the first time interval tested, 3 hours, there was a decrease of over 90% in number of viable particles titratable from blood. This may be in part explainable by the effect of phage neutralizing ability of properdin. Van Vunakis *et al.*(5) have shown this effect on T 2 phage of *Escherichia coli* using sera from mice. In a limited study we verified this effect with this phage strain. It is furthermore noted that 12 hours after inoculation, when no phage particles can be recovered from blood, lung, kidney and brain, virus particles can still be obtained from intestines, liver and in higher amounts from spleen. At 24 hours, viable particles in small amounts could still be recovered from the spleen. That the intestines consistently yielded bacteriophage particles when blood did not, may be explainable by the fact that we have been able to recover phage particles from fecal material collected within 3 to 6 hours after intraperitoneal inoculations of bacteriophage. From the Figures, it is obvious that organs of the reticuloendothelial system such as spleen and liver contain and release phage particles after homogenizing at time intervals when none could be detected in other organs tested.

The results of administration of high titered phage suspensions by gastric lavage are tabulated in Table I. The quantity of viable particles recovered from blood was random and irregular. Because of the wide range of difference in numbers of phage particles recovered at each time interval, the data have been cited for maximum number of phage particles in total blood volume. The proportion of mice which did not yield active particles can possibly be accounted for by the natural immune defenses, *i.e.*, properdin of individual mice, as we have noted wide variations in amount of phage recovered from individual mice after intraperitoneal inoculations of phage preparations. Bacteriophage could be recovered as early as 5 minutes after gastric

TABLE I. Numbers of *B. megatherium* Bacteriophage Recoverable from Blood of Mice after Gastric Lavage.

Time, min.	Max No. of phage particles in total blood vol ($\times 10^8$)	% of mice yielding active virus particles
5	44.0	75
10	3.2	85
15	1.0	60
20	22.0	79
25	16.0	67
30	85.0	77
35	2.0	56
40	19.0	86
45	61.0	67
50	13.0	54
55	8.9	78
60	20.0	77
	Avg %	71.8

Cumulative information of 14 experiments utilizing 135 mice.

lavage. Essentially similar results were noted when the experimental method was varied to administer the phage suspension by oral inoculations. As particles of this size range could pass the gastrointestinal barrier of normal mice, alternative experiments were carried out to obtain information on passage of bacteriophage into the gastrointestinal tract, as measured by assaying fecal material, and through the renal barrier as assayed by quantitating urinary samples. Experiments yielded again an irregular but repeatable amount of positive migration of phage particles. Particles were recovered in urine samples as early as 30 minutes. Fecal samples yielded phage particles within 3 to 6 hours after intraperitoneal inoculation.

Discussion. The results of the translocation studies indicate that *B. megatherium* bacteriophage, whose size as determined by Murphy(6) is 49 mμ for width of head and 330 mμ x 15 mμ for the tail, is able to pass apparently normal anatomical barriers such as gastro-intestinal tract and renal filters. Studies by Gordon *et al.*(7) have shown with feeding experiments of mice utilizing the bacterium *Serratia marcescens,* that organisms of this size in small amounts could pass the intestinal mucosa to the mesenteric lymph nodes and that such bacteria were localized at this site. These same studies cited that bacteria could not be isolated from the blood. It therefore appears that particles the size of bacteria can pass through the lymphatic system, but not through the small capillaries and blood vessels of the gastric mucosa. Whether the bacteriophage particles accounted for in the blood stream in our experiments passed through the lumen of the gut *via* the lymphatic system or blood capillaries was not determined, but on the basis of the short time necessary for the passage it would appear that a direct blood route is more likely. It thus appears that particles of this size range may not respect histological barriers such as the capillary endothelium. In this regard the speculation arises as to whether animal viruses such as the poliomyelitis virus which has been shown to have as one primary area of entrance, the intestines (8), could also enter directly into the blood circulation without prior infection and multiplication within cells of the alimentary mucosa and lymph nodes as is stated in a recent review(9). The results of the experiments dealing with the fate of bacteriophage intraperitoneally inoculated into mice indicate that biological particles of this size range are concentrated in organs of the reticulo-endothelial system such as the liver and spleen, and may in part explain why protection against challenge infections with *Shigella paradysenteriae* could be achieved up to 7 days after phage administration(10). This introduces the problem whether such filter-like organs also have ability to inactivate such localized particles by phagocytosis. A recent report(11) indicates that influenza virus, which has been shown to adsorb and to reduce the anaerobic glycolysis of guinea pig leucocytes(12), can be phagocytized by human and rabbit leucocytes. If phagocytosis of bacterial viruses can also occur and whether such phagocytized phage particles can be released from leucocytes subsequent to artificial breakage of such cells is being considered.

The preliminary information that bacteriophage particles pass through the renal filter has been extended and quantitated in studies with dogs in a separate report(13).

Summary. The fate of *Bacillus megatherium* bacteriophage intraperitoneally inoculated into mice has been investigated. Active bacteriophage particles were recovered from spleen and liver at a time when none were recovered from blood, lung, kidney, and brain. The relationship of this occurrence to possible phagocytosis of bacteriophage particles is discussed. Introduction of bacteriophage suspensions of high titer into the gastro-intestinal tract by feeding and gastric lavages led to an irregular but consistent recovery of active phage particles from the blood circulation. Conversely, phage particles inoculated intraperitoneally were recovered in samples of urine and feces.

1. Nungester, W. J., Watrous, R. M., PROC. SOC. EXP. BIOL. AND MED., 1934, v31, 901.

2. Appelmans, R., *Compt. Rend. Soc. Biol.,* 1921, v85, 722.

3. Gratia, A., *ibid.,* 1936, v123, 1018.

4. Adams, M. H., *Methods Med. Res.,* 1950, v2, 7.
5. Van Vunakis, H., Barlow, J. L., Levine, L., *Proc. Nat. Acad. Sci.,* 1956, v42, 391.
6. Murphy, J. S., *J. Exp. Med.,* 1953, v98, 657.
7. Gordon, L. E., Ruml, D., Hahne, H. J., Miller, C. P., *ibid.,* 1955, v102, 413.
8. Burnet, F. M., Jackson, A. V., *Austral. J. Exp. Biol. Med. Sci.,* 1940, v18, 361.
9. Sabin, A. B., *Science,* 1956, v123, 1151.
10. Morton, H. E., Engley, F. B., Jr., *J. Bact.,* 1945, v49, 245.
11. Boand, A. V., Jr., Kempf, J. E., Hanson, R. J., *J. Immunol.,* 1957, v79, 416.
12. Fisher, T. N., Ginsberg, H. S., *Virology,* 1956, v2, 637.
13. Zatzman, M. L., Keller, R., *The Physiologist,* 1957, v1, 92.

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Provocation and Prevention of Potassium Deficiency by Various Ions.* (24113)

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Earlier work on the Electrolyte-Steroid-Cardiopathy that is characterized by massive, myocardial Necroses (ESCN) showed that, at least in the corticoid-conditioned rat, toxicity of sodium is decisively influenced by: (1) the anion to which Na is attached, (2) concurrent administration of various other cations. Thus, after sensitization with certain corticoids, Na_2HPO_4, NaH_2PO_4, $NaClO_4$ or Na_2SO_4 produced extensive myocardial necroses in rats, while equimolecular amounts of NaCl were ineffective in this respect. Furthermore, the ESCN elicited by corticoids plus sensitizing Na-salts was completely prevented by simultaneous administration of magnesium and potassium ions, particularly when these were given as chlorides. The nephro-calcinosis that normally accompanies the ESCN when the latter is produced with the aid of Na-phosphates was likewise prevented by KCl or $MgCl_2$(1). It has long been known, furthermore, that rats kept either on K-deficient(2, 3,4) or on Mg-deficient(3,5,6) diets also tend to develop focal myocardial necroses and nephrocalcinosis. In the case of Mg-deficiency, these changes are frequently accompanied by convulsions. Several observations suggested that an excess of Na can aggravate K-deficiency(7,8), but in none of these earlier studies were the effects of various Na-salts compared, because at that time there was no reason to suspect a dependence of Na-actions upon anions. The possibility of a replacement of K by Mg was also disregarded. Thus, for example, one group of workers claimed that administration of excess NaCl aggravates the myocardial necroses characteristic of severe K-deficiency(9,10). They arrived at this conclusion by substituting $MgCl_2$ for NaCl in their control rats. Schrader *et al.* (3) postulated that simultaneous Mg-deficiency does not significantly alter the course of K-deficiency in the rat; indeed, some workers believe that there exists an actual antagonism between Mg and K(11,12).

In view of our findings concerning participation of electrolytes in production of myocardial necroses by steroids, we wished to determine: (1) whether the cardiac necroses and other manifestations of dietary K-deficiency would be most markedly aggravated by those Na-salts that sensitize for production of the ESCN, and (2) whether the morbid changes usually ascribed to a lack of K are accentuated by a concurrent deficiency in Mg.

Materials and technics. In the first experiment, 60 female Sprague-Dawley rats, with a mean initial body-weight of 49 g (range 43-55 g), were placed on the "Low Potassium Diet" of the Nutritional Biochemicals Corp. (Cleveland, Ohio) for 7 days. This diet consists of: corn starch 64.2%; casein 30%;

* Supported by Lilly Research Laboratories and by Quebec Asbestos Mining Assn.

TABLE I. Provocation and Prevention of Potassium Deficiency by Various Ions (First Exp.).

Group	Treatment	Cardiac necrosis	Nephro-calcinosis	Muscular cramps	Mortality (%)
1	None	1.1 ± .30	0	0	10
2	Na_2HPO_4	2.7 ± .10	.3 ± .30	3.0 ± 0	100
3	KCl	0	0	0	10
4	KCl + Na_2HPO_4	.1 ± .10	.1 ± .10	Transient tremor	30
5	$MgCl_2$	.4 ± .20	0	0	30
6	$MgCl_2$ + Na_2HPO_4	1.1 ± .30	0	0	20

butterfat 3.5% calcium carbonate 1.3%; sodium chloride 1%; and a vitamin mixture which contains per 100 lb diet: Vit. A concentrate (200,000 units/g) 4.5 g; Vit. D concentrate (400,000 units/g) 0.25 g; alpha tocopherol 5.0 g; ascorbic acid 45.0 g; inositol 5.0 g; choline chloride 75.0 g; menadione 2.25 g; p-aminobenzoic acid 5.0 g; niacin 4.5 g; riboflavin 1.0 g; pyridoxine hydrochloride 1.0 g; thiamine hydrochloride 1.0 g; calcium pantothenate 3.0 g; biotin 20.0 g; folic acid 90.0 mg; Vit. B_{12} 1.35 mg. On 7th day, the rats were subdivided into 6 equal groups and treated as indicated in Table I. 0.5 mM of dibasic anhydrous sodium phosphate, Na_2HPO_4 (Baker), 0.25 mM of potassium chloride, KCl (Fisher) and 0.25 mM of magnesium chloride, $MgCl_2 \cdot 6H_2O$ (Merck)—all "Reagent" degree of purity—were administered in 2 ml of water, twice daily, by stomach tube. The experiment was terminated on the 13th day, that is, after 6 days of electrolyte treatment. In the second experiment, 50 female Sprague-Dawley rats, with a mean initial body-weight of 51 g (range: 43-55 g), were placed on the same "Low Potassium Diet" used in the first series. On the 7th day the rats were subdivided into 5 equal groups and treated as indicated in Table II. The treatment was the same as in the first experiment except that, here, 0.5 mM of Na_2HPO_4 was administered once daily, and 0.5 mM of KCl and of $MgCl_2$ was given twice daily by stomach tube, always in 2 ml of water. Furthermore, a special control group was added which received the same amount of Na, in the form of 1 mM of NaCl, as was given to the rats treated with Na_2HPO_4. This experiment was terminated on the 9th day, that is, after 2 days of electrolyte treatment. In both experimental series the heart and kidneys were fixed in neutral formalin and stained with the acid-fuchsin technic(1) for demonstration of early necrotic changes, and with von Kossa's silver nitrate technic for histochemical detection of calcium. Both cardiac necroses and the nephrocalcinosis have been assessed in terms of an arbitrary scale of 0-3, the means of these readings (with standard errors) being indicated in our Tables.

Results. The most striking change observed in the course of the *first experiment* was the development of intense muscular cramps and convulsions, in the Na_2HPO_4-treated rats (Group 2). There was at first a fine fibrillar tremor followed by slow spastic extensions of the hind paws, and, eventually, generalized convulsions with forceful extensor movements in both hind legs. These cramps began 2-3 hours after Na_2HPO_4 administration and resulted in 100% mortality within the first 24 hours. Slight transient tremor was also observed in the rats which, in addition to Na_2HPO_4, received KCl (Group 4) but in none of the other groups.

Yellowish patches of myocardial necroses were quite evident, even by mere macroscopic inspection, in most of the animals of Group 2. The histologic findings (Table I) indicate that after having been kept on the "Low Potassium Diet" for 13 days, even the otherwise untreated controls (Group 1) showed moderate cardiac necroses. These lesions were significantly aggravated by Na_2HPO_4 (Group 2), but prevented or diminished by KCl (Group 3) or $MgCl_2$ (Group 5). Indeed, even in the event of simultaneous treatment with Na_2HPO_4, both KCl (Group 4) and $MgCl_2$ (Group 6) exerted at least a partially protective effect upon the induction of cardiac necroses.

Nephrocalcinosis was negligible in all groups of this series, presumably because the animals most likely to develop it (those of

TABLE II. Provocation and Prevention of Potassium Deficiency by Various Ions (2nd Exp.).

Group	Treatment	Cardiac necrosis	Nephro-calcinosis	Muscular cramps	Mortality (%)
1	None	0	0	0	0
2	NaCl	0	0	0	0
3	Na_2HPO_4	$2.5 \pm .20$	$2.8 \pm .10$	3.0 ± 0	60
4	$KCl + Na_2HPO_4$	0	0	Transient tremor	0
5	$MgCl_2 + Na_2HPO_4$	0	0	0	0

Group 2) died within a few hours after the Na_2HPO_4 treatment. The detrimental effect of Na_2HPO_4 can definitely be ascribed to conditioning by the "Low Potassium Diet," because numerous control experiments(1) showed that, under similar circumstances, even fatal doses of Na_2HPO_4 produce no cardiac necroses or muscular cramps.

In the second experiment, Na_2HPO_4 treatment was reduced while the prophylactic administration of KCl and $MgCl_2$ was increased. This was done, in order to prolong survival and, thereby, permit development of morphologic changes in the rats treated with Na_2HPO_4 and, at the same time, to offer a better chance of protection by KCl and $MgCl_2$. The results summarized in Table II indicate that, under these conditions, marked cardiac necroses and nephrocalcinosis developed after 2 days of Na_2HPO_4 treatment following 9 days on the "Low Potassium Diet"; at this time the controls (Group 1) had not yet developed cardiac necroses. The experiment also shows that Na_2HPO_4 was not effective merely by virtue of its Na-content, since equivalent amounts of NaCl (Group 2) were completely ineffective. On the other hand, the somewhat larger doses of KCl and $MgCl_2$ given in this experiment offered virtually complete protection against the mortality as well as the cardiotoxic, nephrotoxic and muscular-cramp-producing effects of combined treatment with the "Low Potassium Diet" plus Na_2HPO_4.

Discussion. The earlier findings reported in our introduction created the impression that Mg-content of the diet is of little if any importance in determining course of K-deficiency(3). This is presumably why the Nutritional Biochemical Co. (Cleveland, Ohio), which prepares the "Low Potassium Diet"—that we, like so many other laboratories, have used—did not consider it necessary to check the Mg-content of this ration. We found it to contain 52 mg/kg, which is close to the minimal amount necessary for growth according to Tufts and Greenberg(13). On the other hand, the latter investigators claim to have demonstrated, by adding various concentrations of calcium phosphates to Mg-deficient diets, that "a high content of calcium in the diet increases the severity of magnesium deficiency." However, they disregarded the fact that when Ca was raised (from 0.39 to 1.66%), phosphorus was also considerably increased (from 0.45 to 1.00%). Although it is quite possible that Ca itself exerts a sensitizing effect, it is also evident from our experiments that PO_4 can do so even when not attached to Ca.

Summary and conclusions. When rats are kept on a "Low Potassium Diet" that contains only near minimal maintenance levels of Mg, there develop cardiac necroses which can be prevented both by KCl and by $MgCl_2$ administration. On the other hand, Na_2HPO_4 (unlike equivalent amounts of NaCl) rapidly provokes development of severe cardiac necroses, nephrocalcinosis and muscular cramps before this diet, in itself, produces any obvious morbid changes. The particularly severe K and/or Mg-deficiency syndrome induced by Na_2HPO_4– supplements, in animals on this diet, can be prevented by either KCl or $MgCl_2$. These observations highlight the importance of PO_4– and Mg-ions in the development of the syndrome usually ascribed to K-deficiency.

We are greatly indebted to Dr. K. Mori for determination of the Mfg-content of the diet used in these experiments.

1. Selye, H., *The Chemical Prevention of Cardiac Necroses,* The Ronald Press Co., N. Y., 1958.

2. Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.,* 1942, v71, 235.

3. Schrader, G. A., Prickett, C. O., and Salmon,

W. D., *J. Nutrition,* 1937, v141, 85.

4. Thomas, R. M., Mylon, E., Winternitz, M. C., *Yale J. Biol. and Med.,* 1940, v12, 345.

5. Greenberg, D. M., Anderson, C. E., Tufts, E. V., *Proc. 30th Ann. Meet. Am. Soc. Biol. Chem.,* 1936.

6. Lowenhaupt, E., Schulman, M. P., Greenberg, D. M., *A.M.A. Arch. Path.,* 1950, v49, 427.

7. Meyer, J. H., Grunert, R. R., Zepplin, M. T., Grummer, R. H., Bchstedt, G., Phillips, P. H., *Am. J Physiol.,* 1950, v162, 182.

8. Freed, S. C., Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 74.

9. Cannon, P. R., Frazier, L. E., Hughes, R. H., *Metabolism,* 1953, v2, 297.

10. Rahman, M. H., Frazier, L. E., Hughes, R. H., Cannon, P. R., *A.M.A. Arch. Path.,* 1957, v63, 154.

11. Colby, R. W., Frye, C. M., *Am. J. Physiol.,* 1951, v166, 209.

12. Smith, S. G., *ibid.,* 1951, v164, 702.

13. Tufts, E. V., Greenberg, D. M., *J. Biol. Chem.,* 1938, v122, 715.

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Adenovirus Vaccine Evaluation Study in Naval Recruits.*† (24114)

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Acute respiratory disease in military inductees continues to be a major problem (1,2,3). During the past 4 years adenoviruses, particularly types 3, 4 and 7, caused 20 to 70% of acute respiratory tract infections among military recruits(3,4,5,6,7,8). Several studies employing polyvalent adenovirus vaccines for prevention of respiratory disease have been reported(9,10,11,12,13). In their first study, Hilleman and associates evaluated a formalin-inactivated monkey kidney culture vaccine composed of types 4 and 7 adenoviruses, prepared at Army Medical Center. A subsequent study was made by them of vaccine prepared commercially(13).‡ Bell and associates used commercially prepared§ kidney cell culture vaccine containing adenoviruses types 3, 4 and 7(12). All studies have shown these vaccines highly effective in preventing infections due to types 4 and 7 adenoviruses, the only prevalent types isolated from recruit populations.

The present report gives results of an adenovirus vaccine evaluation study among inductees at Naval Training Center at San Diego, Calif., where both types 4 and 7 adenovirus infections were prevalent in the population, from March 11 to July 3, 1957.

Materials and methods. Vaccine was prepared by Eli Lilly and Co. Seed adenoviruses, types 3, 4 and 7, obtained from Dr. Robert J. Huebner at N.I.H., where they had been isolated directly from humans in monkey kidney cell cultures. Pools of each virus type were grown in 16 ounce bottles of trypsinized monkey kidney cells. After filtration each pool was inactivated with formaldehyde (1:4000) at

* These investigations were conducted under sponsorship of the Commission on Influenza, Armed Forces Epidemiological Board and supported by Office of Surgeon General, Dept. of Army; and by grants from Eli Lilly and Co., Common Cold Fn., and Seymour Coman Fellowship Fund, Univ. of Chicago.

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‡ Lederle Laboratories.

§ Parke, Davis and Co.

37°C for 7 days. These pools were then neutralized by addition of sodium bisulfite. The trivalent vaccine was prepared by mixing equal parts of 3 monovalent types after which merthiolate 1:20,000 was added as preservative. Before mixing and inactivation each pool of virus was titered and tested with specific antiserum for purity and identification. Control procedures on the pools and trivalent vaccine were those employed for safety testing of poliomyelitis vaccine as recommended by U. S. Public Health Service. *Placebo*: Tissue culture maintenance medium to which formaldehyde (1:4000) and preservative had been added was employed. One ml of vaccine or placebo material was given intramuscularly to test and control groups. Population study comprised personnel of 39 recruit training companies activated at Training Center from Mar. 11 to May 3. Vaccine and placebo were administered within a day or two after the company was formed. Half the men in a company were given adenovirus vaccine while the other half received placebo material. Selection of vaccine and controls was determined by final digit of recruit's service number. Individuals with even numbers were given the vaccine, those with odd numbers received control material. The vaccinated group totaled 1,203 and the control group 1,249. Of these, 132 in the test and 151 in the control group were discharged some time subsequent to one week and prior to completion of ninth and final week of training. *Criteria for evaluation of vaccine*: Observations were made on study companies until the last had completed training July 6, 7 and 8. Effectiveness of vaccine was based on Dispensary admissions for adenovirus infections (based on laboratory data) from vaccinated and control groups. On each admission temperature, day and month and weeks of training were recorded along with individual's name and unit number on punch cards. *Laboratory procedures*: Throat swabs and washings with nutrient broth and acute and convalescent sera were collected on essentially all admissions up to seventh week of training. Some throat washings were collected on individuals during 8th and 9th weeks. Throat swabs with washings were frozen within an hour. Bloods were brought to Unit laboratory where serums were separated and frozen. Throat washings and paired serums were shipped at bi-weekly intervals under dry ice *via* air express to University of Chicago where they were analyzed for presence of adenoviruses and, in certain specimens, for influenza viruses, and complement fixing antibodies with adenovirus and influenza virus antigens. Hemagglutinin inhibiting (H.I.) antibodies were also determined on all paired sera employing A′/Denver/57, A/Asian/57 and B/Great Lakes/54 influenza antigens. For adenovirus isolation test tube cultures of HeLa cells were employed. Details of methods used have been published(14). Fertile egg technic was employed for isolation of influenza viruses(15). Complement fixation test for determining adenovirus and influenza antibodies was that described by Jensen(15). Antigen for complement fixation test for adenovirus was prepared by pooling cultures of adenoviruses, types 1 through 7. The hemagglutination inhibition antibody test was that recommended by Committee on Standard Serological Procedures of Armed Forces Epidemiological Board(16). For influenza antibody determinations allantoic fluid harvest, undiluted and uninactivated, of Jap/305/57, A′/Denver/57 and B/Great Lakes/57 were employed as antigens. Rapid neutralization test and colorimetric assay method for determination of adenovirus antibodies were used. These procedures have been described(17, 18, 19).

Results. Detailed differential clinical study of cases admitted to Dispensary was not made. Cases were admitted generally because they had symptoms of acute respiratory disease and were febrile with temperatures of 100°F or greater. Diagnoses of adenovirus and influenza infections were made on basis of virus isolation and complement fixing or H. I. antibody responses.

Adenovirus infections in non-study population. Approximately 50 throat washings and paired bloods were collected weekly. In the beginning many were from individuals admitted to Dispensary from companies which were on board before the vaccine study began. From week ending Mar. 10 to May 5 the

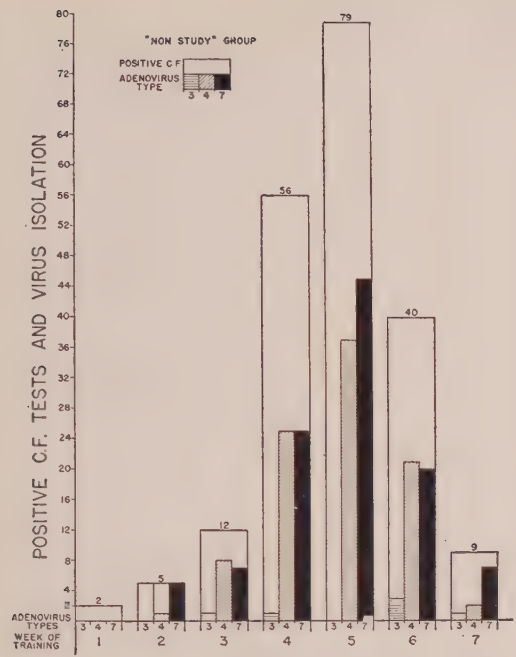


FIG. 1. Adenovirus isolation and positive complement fixation tests in relation to week of training of individuals in "non-study" companies.

weekly adenovirus rate/1,000 from this "non-study" group was 15.8 and interpolated weekly admission rate/thousand due to adenovirus was 12.6. Thus, 80% of admissions during this period were due to adenovirus infections. Fig. 1 shows adenovirus isolations from 265 throat washings and complement fixing antibody responses in 244 sets of serums from individuals from "non-study" companies recorded in relation to week of training. Two hundred and three (81%) of the paired serums showed 4-fold or greater rises with adenovirus pooled antigen. Two hundred and five (79%) of 265 throat washings yielded adenoviruses, either types 3 (6), 4 (94) or 7 (105). The great majority with adenovirus infections were admitted during fourth, fifth and sixth weeks of training. Only a few were admitted during second, third and seventh weeks. Throat washings and sera were not collected during 8th and final 9th week of training.

Antigenicity studies. Recruits of 2 companies at beginning and 3 at end of study were bled before and at 14 days after vaccination to determine antibody response to the vaccine. Paired serums selected at random from each company were tested for complement fixing and neutralizing antibody response with types 3, 4 and 7 adenovirus antigens. The results are shown in Table I. Adenovirus infections in the placebo group were low, as indicated by low complement fixing and neutralizing antibody response to the 3 antigens. Eight of 81 individuals in the vaccine group showed 4-fold or greater rise in complement fixing antibodies. There was good neutralizing antibody response to types 3 and 7 adenovirus antigens. However, only 26 or 31% of 85 individuals showed significant antibody response to type 4 antigen. This was of interest for the vaccine was not as effective against type 4 adenovirus infections as against type 7.

Adenovirus isolation. As seen in Table II, during the first 2 weeks post vaccination, throat washings were taken on 95% of admissions from the vaccine group and 82% from control group. Only 2 adenoviruses, one type 4 and one type 7, were obtained from vaccinated individuals. None was obtained from

TABLE I. Adenovirus Antibody Response following Vaccination with a Polyvalent Adenovirus Vaccine Containing Types 3, 4 and 7.*

	Vaccine group				Control group			
		Neutralization test with adenovirus types				Neutralization test with adenovirus types		
Co.	C.F. test	3	4	7	C.F. test	3	4	7
125	0/16	13/16	4/15	11/16	1/20	1/20	1/20	1/20
130	3/22	20/27	10/27	22/27	0/21	0/24	1/24	0/24
156	1/12	8/11	5/12	7/12	1/ 7	1/ 5	1/ 7	0/ 7
157	2/14	7/14	3/14	6/12	0/11	2/11	2/11	1/ 9
158	2/17	12/17	4/17	10/17	0/ 9	0/ 9	0/ 8	0/ 8
Total	8/81	60/85	26/85	56/84	2/68	4/69	5/70	2/68
	10%	71%	31%	67%	3%	6%	7%	3%

* No. showing 4-fold or greater antibody rise/No. tested.

TABLE II. Adenovirus Isolation and Complement Fixation Antibody Response in Vaccine and Control Groups.

	2 wk post-vaccination		3 thru 9 wk post-vaccination	
	Vaccine	Control	Vaccine	Control
Avg weekly population	1197	1242	1168	1210
Admissions, total	38	27	184	221
Throat washings	36	22	122	168
% admissions	95	82	66	76
Adenovirus isolation	2	0	16	69
% isolation	5.2	0	13.1	41.0
Adenovirus types				
3	0	0	1	5
4	1	0	13	36
7	1	0	2	28
Paired sera	36	22	103	139
% admissions	95	82	56	63
C. F. positive	10	6	15	69
% positive	27	27	14.6	47.5

unvaccinated individuals. During 3 through 9 week post-vaccination period throat washings were obtained on 66% of admissions from the vaccine group and 76% from control group. Only 13.1% of throat washings from the vaccinated group yielded adenovirus viruses, while adenoviruses were obtained from 41% of throat washings from unvaccinated individuals. These data and the adenovirus types isolated are also shown in Table II. Of 16 adenoviruses isolated from vaccinated individuals, one was type 3, 13 were type 4, and 2 were type 7. The 69 adenoviruses from the control group comprised 5 type 3, 36 type 4 and 28 type 7.

Adenovirus antibody response to infection. Complement fixing adenovirus antibody rises in admitted cases from vaccinated and control groups are shown in Table II. During the first 2 weeks of training, 27% of paired serums from both groups showed 4-fold or greater complement fixing antibody rises to the adenovirus antigen. Fourteen % of paired serums from vaccinated individuals admitted during 3 to 9 week period showed significant rises compared to 47% from unvaccinated individuals. Distribution of virus isolation and complement fixation antibody rises in individuals according to week of post vaccination, is shown in Fig. 2. In the vaccine group the greatest number of infections occurred during 5th, 6th and 7th week post vaccination, all due to type 4 adenovirus except for one type 3 and one type 7. In the control group the greatest number of infections also occurred during these weeks while a moderate number also occurred during third and eighth week of training.

Dispensary admissions from vaccine and control groups due to adenoviruses. During the first 2 weeks post-vaccination the weekly admission rate/1,000 was 15.8 from the vaccine group and 10.8 from control group (Table III). During the 3 through 9 post-vaccination period, weekly admission rate was 22.3 from the vaccine group and 26.2 from control group. This represents a difference of 15% in favor of the vaccine group. When admissions due to adenoviruses were determined

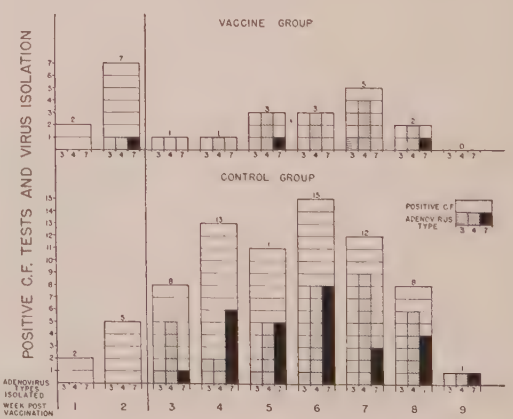
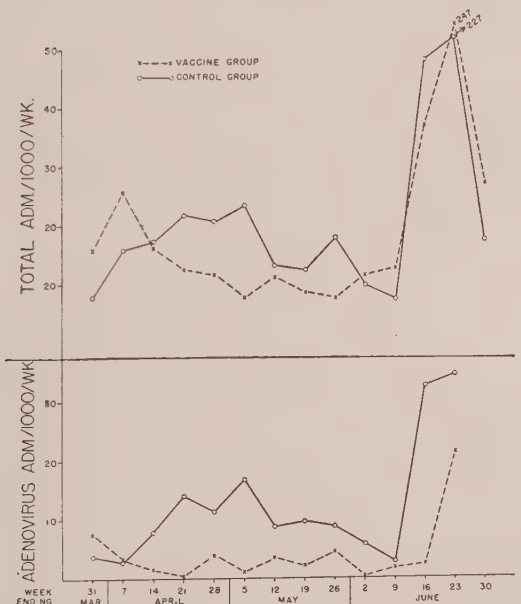


FIG. 2. Adenovirus isolation and complement fixing antibody response in vaccinated and unvaccinated individuals in relation to week post-vaccination.

TABLE III. Total Admissions and Admissions Due to Adenovirus Infections from Vaccine and Control Groups.

	2 wk post-vaccination		3 thru 9 wk post-vaccination		%
	Vaccine	Control	Vaccine	Control	difference
Avg weekly population	1197	1242	1168	1210	
Admissions, total	38	27	184	221	
Admission rate/1000/wk					
Total	15.8	10.8	22.3	26.2	15
By adenovirus isolation	1.0	0.0	3.4	10	66
By C. F. antibody rises	4.8	2.8	3.4	12	72

from laboratory data, including virus isolation and complement fixation antibody response, admission rates were significantly lower in the vaccine group. On the basis of adenovirus isolations, during 3 to 9 week post-vaccination period, weekly admission rate was 3.4 from the vaccinated group compared to 10 from control group. This represents a difference of 66%. When 4-fold or greater complement fixation antibody responses to adenovirus antigen were employed as evidence of infection the weekly admission rate was 3.4 from the vaccinated group, and 12 from the control group, a difference of 72% in favor of the vaccine. Weekly admission rates from vaccine and control groups are also shown in Fig. 3. The weekly adenovirus admission rate after April 7, 4 weeks after vaccination began, was consistently lower from the vaccinated group. The sharp rise in total admissions from both vaccinated and control groups during June represents Asian influenza infections as shown below. There was also a corresponding rise in adenovirus admission rates in both vaccine and control groups during this time. Weekly admission rates according to week post-vaccination are shown in Fig. 4. After the second week, admissions for adenovirus infections from the vaccinated group were low and remained so except for 7th and



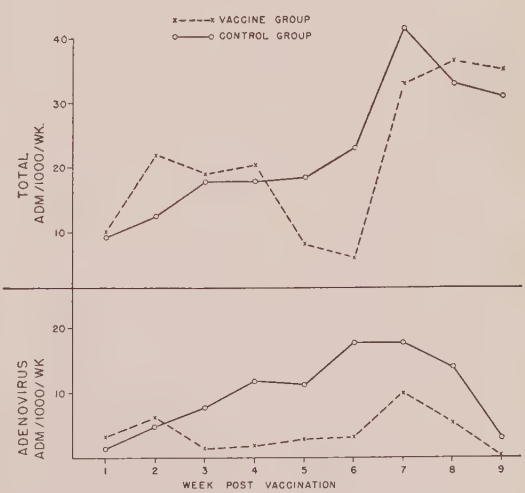


FIG. 3 (left). Total and adenovirus infection admission rates from vaccine and control groups.

FIG. 4 (right). Total and adenovirus infection weekly admission rates in relation to week post-vaccination.

8th week of training. Increase in total admissions during 7th, thru 9th week represent admissions due to Asian influenza.

Influenza infections. During the latter part of the investigation an outbreak of influenza occurred at the Training Center. To identfy the increased admission rate as influenza, complement fixing antibody determinations were carried out on paired sera from vaccine and control groups using A/Jap/305/57 and B/GL/54 as antigens. Paired serums having 4-fold or greater rises were then tested for H. I. antibodies with A'/Denver/57, A/Jap/305/57 and B/GL/57 antigens. Thirty-two % of sera from the vaccinated group and 27% from control group showed significant complement fixing antibody rises with A and B antigens. With the H.I. procedure the majority of influenza infections were found to be due to Asian influenza viruses.

Discussion. This study confirms those of others that adenovirus infections are a major cause of acute respiratory disease in military inductees. Eighty % of admissions from "non-study" companies admitted to the Dispensary from Mar. 5 to May 5 were due to either type 3, 4 or 7 adenoviruses. Forty % of control population of study companies March 31 to July 3 were also admitted because of adenovirus infection due to one of the above 3 types. During this same period only 15% of admissions from vaccinated group were due to adenovirus infections. As periods of observation were not the same, it cannot be assumed that the 40% admission rate in the 50% vaccinated companies compared to an 80% admission rate from the "non-study" companies was due to "herd immunity" effect.

The vaccine was effective against all 3 types as indicated by differences in numbers of 3, 4 and 7 adenoviruses isolated from vaccine and control groups. Of 16 adenoviruses isolated after second week from vaccinated individuals 13 were type 4. This was of interest for antibody response studies showed that the vaccine elicited significant rises to type 4 antigen in only one-third of the individuals. Thus, the isolation of type 4 viruses from vaccinated individuals may reflect a failure on the part of the vaccine to produce a significant protective antibody response.

The overall reduction in admission rates for adenovirus infection was 66% when virus isolations were used as a criterion and 72% when complement fixing antibody response was employed. These results compare favorably with previous studies reported by Hilleman and associates(13) and Bell and colleagues(12). In a controlled field study at Fort Dix, N. J., Hilleman obtained 98% reduction in acute respiratory disease due to adenoviruses(9,11) by use of type 4 or 7 adenovirus vaccine. In other studies(8,13) at Fort Leonard Wood, Mo., during 1957 employing bivalent types 4 and 7 adenovirus vaccine, adenovirus infection was reduced by 90%. At Great Lakes Bell, Hantover, and colleagues found polyvalent types 3, 4 and 7 adenovirus vaccine reduced total febrile respiratory disease incidence by 55%, and 65% of such illnesses requiring hospitalization during third to ninth week of training(12).

In addition to adenovirus infections, influenza infections occurred in study population with equal frequency in test and control groups.

Conclusions. 1) An adenovirus vaccine evaluation study was carried out at San Diego Naval Training Center. Recruit population was studied. Kidney cell tissue culture vaccine containing adenovirus types 3, 4 and 7 was used, inactivated with formalin. Half of the men in 39 companies were given vaccine while the other half were given placebo. Observations were made from March 11 to July 3, 1957. Adenovirus isolations and complement fixing antibody response in acute and convalescent serums were employed as indices of infection. Types 4 and 7 adenovirus infections were prevalent in the control population. 2) During 3 through 9 week post-vaccination period, weekly admission rate/1,000 was 22.3 from the vaccine group, and 26.2 from the control group, a 15% difference. However, on the basis of adenovirus isolation the weekly admission rate was 3.4 from the vaccinated compared to 10 from the control, a difference of 66%. When 4-fold or greater complement fixing antibody rises to adenovirus antigen were employed as evidence of infection, the weekly admission rate was 3.4

from the vaccinated group and 12 from the control group, a difference of 72% in favor of the vaccine. On the basis of above findings, adenovirus vaccine significantly reduced number of adenovirus infections in the vaccinated group compared to the control group. 3) In non-study companies from Mar. 5 to May 5 adenovirus infections accounted for 80% of Dispensary admissions. Forty % of admissions from the control group of the study companies were due to either types 3, 4 or 7 adenovirus while only 15% from the vaccine group were due to these agents.

1. Sartwell, P. E., *Am. J. Hyg.,* 1951, v53, 224.
2. Seal, J. R., *Military Med.,* 1955, v116, 265.
3. Duff, F. L., *U. S. Armed Forces Med. J.,* 1956, v7, 937.
4. Hilleman, M. R., Werner, J. H., Adair, C. V., Dreisbach, A. R., *Am. J. Hyg.,* 1955, v61, 163.
5. Berge, T. O., England, B., Shuey, H. E., Lennette, E. H., *ibid.,* 1955, v62, 283.
6. Rowe, W. P., Seal, J. R., Huebner, R. J., Whiteside, J. E., Woolridge, R. L., Turner, H. C., *ibid.,* 1956, v64, 211.
7. Woolridge, R. L., Grayston, J. T., Whiteside, J. E., Loosli, C. G., Friedman, M., Pierce, W. E., *J. Inf. Dis.,* 1956, v99, 182.
8. Hilleman, M. R., *Ann. N.Y. Acad. Sci.,* 1957, v67, 262.
9. Hilleman, M. R., Stallones, R. A., Gauld, R. L., Warfield, M. S., Anderson, S. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 377.
10. Hilleman, M. R., Warfield, M. S., Anderson, S. A., Werner, J. H., *J.A.M.A.,* 1957, v163, 4.
11. Stallones, R. A., Hilleman, M. R., Gauld, R. L., Warfield, M. S., Anderson, S. A., *ibid.,* 1957, v163, 9.
12. Bell, J. A., Hantover, M. J., Huebner, R. J., Loosli, C. G., *ibid.,* 1956, v161, 1521.
13. Hilleman, M. R., *Am. J. Public Health,* 1958, v48, 153.
14. Grayston, J. T., Loosli, C. G., Smith, M., McCarthy, M. A., Johnston, P. B., *J. Inf. Dis.*
15. Jensen, K. E., *Influenza,* Chap. 8, pp241-262. Diagnostic Procedures for Virus and Rickettsial Diseases. Am. Public Health Assn., 1956.
16. Committee on Standard Serological Procedures in Influenza Studies. *J. Immunol.,* 1950, v65, 347.
17. Grayston, J. T., Johnston, P. B., Smith, M. E., Loosli, C. G., *J. Inf. Dis.,* 1956, v99, 188.
18. Grayston, J. T., Loosli, C. G., Johnston, P. B., Smith, M. E., Woolridge, R. L., *ibid.,* 1956, v99, 199.
19. Johnston, P. B., Grayston, J. T., Loosli, C. G., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 338.

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Epidemic Asian A Influenza in Naval Recruits.*† (24115)

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Asian influenza was first recognized in the United States in mid-June, 1957(1). One of the first outbreaks occurred among recruits at Naval Training Center at San Diego, Calif. (2). At time of outbreak a study of evaluation of an adenovirus vaccine was being made (3). During the week ending June 15 a sharp rise in admission rates for acute respiratory disease occurred in both non-study and study companies. From the study companies the admissions came from both adenovirus vaccinated and placebo vaccinated groups. The illnesses resembled epidemic influenza. As it had been pointed out by Hilleman(4,5) that this age group appeared to be non-immune to the new Asian strain of influenza, it seemed

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important to make a detailed laboratory study of this outbreak to determine extent of morbidity among recruit population.

Materials and methods. Population comprised recruits at Naval Training Center. The study began the week ending May 18th and continued until Dec. 21, 1957. *Laboratory procedures.* Approximately 50 throat washings and paired serums were being collected weekly in connection with the adenovirus vaccine study(3). During the first 3 weeks of the outbreak several dozen samples were taken by personnel of Navy Preventive Medicine Unit #5. Collection of throat washings and sera are described in the previous report(3). They were sent to University of Chicago where they were analyzed respectively for influenza viruses and complement fixing antibodies with influenza A/Jap/305/57 and B/GL/54 antigens. *Serological procedures.* Hemagglutinin inhibiting (H. I.) antibodies were determined on selected sera employing A′/Denver/57, A/Jap/305/57 and B/GL/54 influenza virus antigens. The H. I. antibody test was that recommended by Committee on Standard Serological Procedures of Armed Forces Epidemiological Board(6). The complement fixation test for determining adenovirus and influenza antibodies was that described by Jensen(7). For influenza virus antigens, undiluted and uninactivated allantoic fluid harvests of Jap/305/57, A′/Denver/57 and B/GL/54 were employed. Pooled HeLa cell tissue cultures of adenoviruses, types 1 through 7, were employed as antigen in the complement fixation test. *Virus isolations.* The fertile egg technic was employed for virus isolation as described by Jensen(7). Newly isolated influenza viruses were typed by hemagglutinin inhibition (H.I.) test with known positive A/Jap/305/57, A′/Denver/57 and B/GL/54 antisera.‡ In addition all strains were tested with influenza A PR8 ferret antiserum to rule out laboratory contaminants. No PR8 strains were isolated.

Results. Epidemiological investigation revealed that recruit companies, service schools and ship company personnel were affected from the onset. Clinical findings were those

‡ Kindly supplied by Dr. Keith Jensen.

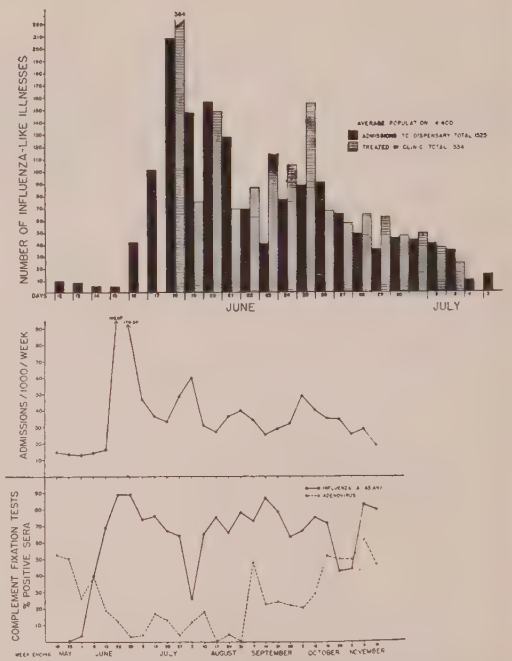

FIG. 1 (top). Daily dispensary admissions and non-admissions from the recruit population for influenza-like illnesses.

FIG. 2 (bottom). Weekly admission rates from the recruit population and per cent positive serums for antibody rise with influenza A (Asian) and adenovirus antigens.

of epidemic influenza. Symptoms occurred abruptly and consisted of fever (100-104°F), chilliness, malaise, frontal headache, myalgia, mild cough, scratchy throat with mild rhinitis and occasional nausea without diarrhea. Physical examination revealed general flushing of skin over face and chest. There was mild conjunctivitis. Throat was diffusely red but not particularly sore. Coughing was not pronounced, and there were essentially no abnormal pulmonary findings.

Daily admissions and visits to Dispensary of recruits for acute respiratory disease from June 12 to July 5, 1957, are shown in Fig. 1. The explosive spread of the infection among recruit population is shown by rapid daily increase in admissions to the Dispensary after the onset. The high daily admission rate continued for 16 days and thereafter gradually fell to a low level by July 5.

The admission rate/1,000 for acute respiratory disease during week ending June 15 was 16.04. During week ending June 22 it was

TABLE I. Influenza Virus Isolation and Antibody Response in Naval Recruits at San Diego Naval Training Center, June 1 to July 20, 1957.

Week ending	Adm. rate per 1000	Throat washings			Paired sera (C.F. test)				
						Type A		Type B	
		Total	No.	% pos.	Total	No.	% pos.	No.	% pos.
June 8	14.47	16	0	0	13	5	38	0	0
15	16.04	33	3	9	23	16	69.5	1	4.3
22	192.19	127	73	57	90	79	88.0	3	3.3
29	170.58	43	23	53	61	54	88.5	1	1.6
July 6	46.83	13	2	15	23	17	74	0	0
13	36.07	15	2	13	29	22	76	0	0
20	33.41	13	3	23	22	15	67	0	0

192.19. The following week it was 170.58. By the week ending July 5 it was 46.83. From June 8 to July 5 approximately 75% of the recruit population either were admitted to or treated in the Dispensary for acute respiratory illnesses resembling epidemic influenza. Figs. 1 and 2.

Laboratory diagnosis. Throat washings and paired sera collected during week ending June 8 through July 20, were examined respectively for influenza viruses and complement fixing antibody response with influenza virus antigens A/Jap/305/57, and B/GL/54. Table I shows that while no viruses were isolated during week ending June 8, five of 13 paired serums showed significant antibody rises with A antigen. During week ending June 22, the period of highest admission rate, 57% of throat washings yielded Asian A influenza viruses and 88% of paired sera showed 4-fold or greater complement fixing antibody rises with type A/Jap/305/57 antigen. During this week one strain of influenza B virus was isolated. Hemagglutination inhibition tests employing Asian A (Jap/305) as antigen showed that essentially all antibody rises were due to this strain. A few showed antibody rises with influenza B antigen.

As seen in Fig. 2 during the summer and fall months, the weekly admission rates for acute respiratory disease continued 2 to 3 times higher than they were during the pre-epidemic period. After the first week in October they declined steadily. Serological studies on paired sera (20/week) for complement fixing antibody rises to influenza A (Asian) and B antigens showed that the majority of admissions following the epidemic outbreak were influenza A infections. A few were due to influenza B infections. Also, many showed complement fixing antibody rises with adenovirus antigen. The number showing rises with adenovirus antigen was low during June, July and August but rose during September and was highest from middle of October through November 16. After this time serological studies were discontinued because an influenza vaccination program was begun.

In Table II are given results of Asian A virus isolation and complement fixing antibody rises in paired sera with Asian A antigen from May through December. Approximately 75% of the paired serums tested during June through November showed rises with Asian A antigen. During August through December selected throat washings of individuals whose serums showed complement fixing antibody rises were tested for Asian A influenza viruses. The results are also shown in Table II. Five of 7 specimens collected during last 2 weeks in August, one of 7 collected during September, 6 of 10 collected during October, 3 of 10 collected during November and 3 of 3 collected during the first 2 weeks in December yielded Asian influenza viruses.

Antibody response survey. The personnel of 3 companies receiving adenovirus vaccine had been bled May 29, 30 and 31, two weeks before outbreak of influenza. They were again bled July 8, approximately 4 weeks after epidemic began. H. I. tests on paired serums for the presence of antibody rises with influenza Asian A antigen were carried out. The results are shown in Table III. It can be seen that 95% of individuals admitted to Dispensary for acute respiratory disease showed

TABLE II. Influenza Virus (Asian A) Isolation and Complement Fixing Antibody Response to Asian A Antigen.

Mo	Avg adm. per 1000 per wk	Throat washings			C. F. antibody rises to Asian A antigen		
		Total	No. pos.	% pos.	Total	No. pos. A	% pos. A
May	13.36	nd	nd		63	0	16
June	98.32	219	99*	45	199	159	80
July	44.89	41	7	15	122	76	62
Aug.	33.48	7†	5	71	85	61	72
Sept.	29.57	7	1	14	80	59	74
Oct.	36.87	10	6	60	86	57	66
Nov.	22.47	10	3	30	17	14	82
Dec.	17.15	3	3	100	nd	nd	

* One type B also isolated.
† Spot check throat washings from persons showing a rise in complement fixing titer to type A.
nd = not done.

TABLE III. Asian Influenza H.I. Antibody Rises in Individuals from 3 Companies of Recruits Bled 2 Weeks before and 3 Weeks after Outbreak of Influenza at San Diego.

Co.	Individuals	Adm. to dispensary		4-fold or greater rise		Non-adm. to dispensary		4-fold or greater rise	
		No.	%	No.	%	No.	%	No.	%
156	44	15	34	13	87	29	66	19	66
157	53	19	36	19	100	34	64	30	88
158	56	23	43	22	96	33	61	24	73
Total	153	57	37	54	95	96	63	73	76

antibody rises. Of individuals not admitted to Dispensary, 76% showed antibody rises. These serological findings confirm the high morbidity of Asian influenza in the recruit population at San Diego during early part of epidemic.

Summary. 1. An explosive outbreak of Asian influenza beginning the week ending June 15, occurred at San Diego Naval Training Center. The peak admission rate of 192.2 from the recruit population occurred the following week. After the first 3 weeks the rates subsided but continued to be 2 to 3 times the pre-epidemic levels through the first 2 weeks in November. Serological and virus isolation studies showed that approximately 75% of admissions from onset of epidemic through November were due to Asian A influenza infections. A few were due to influenza B infections. Admissions due to adenoviruses occurred also in increasing numbers during Sept., Oct. and Nov. On the basis of serological studies essentially all admissions to the Dispensary from onset of epidemic to Nov. 16 were due either to influenza or adenovirus infections. 2) The general lack of immunity to Asian influenza virus in the recruit population is reflected in the high attack rate during the first 3 weeks of the outbreak and high percentage of individuals showing an antibody response to Asian A antigen in the 3-survey companies bled before and 4 weeks after the onset of the epidemic.

1. *Morbidity and Mortality Weekly Report, USPHS,* 6:#26, July 6, 1957.
2. *Ibid.*, 6:#28, July 19, 1957.
3. Loosli, C. G., Tipton, V. C., Warner, O., Smith, M., Johnston, P. B., Hamre, D., to be published.
4. Hilleman, M. R., Meyer, A. M., *Morbidity and Mortality Weekly Report, USPHS* 6:#21, May 31, 1957.
5. Meyer, H. M., Jr., Hilleman, M. R., Messe, M. L., Crawford, I. P., Bankhead, A. S., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 609.
6. Committee on Standard Serological Procedures in Influenza Studies. *J. Immunol.,* 1950, v65, 347.
7. Jensen, K. E., *Influenza,* Chapter 8, pp241-262, American Public Health Association, 1956.

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Failure of 17α-Ethyl-19-Nortestosterone to Effect Plasma 17-Hydroxycorticosteroids and ACTH Responsiveness in Man.* (24116)

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17α-ethyl-19-nortestosterone (Nilevar) has been reported to cause suppression of adrenocortical secretion(1,2). Brooks and Prunty administered 50 to 100 mg of 17α-ethyl-19-nortestosterone daily for 7 to 15 days to 4 men and one woman. Four subjects received the medication intramuscularly and one orally. They found significant reduction in the 17-ketosteroid excretion in all patients; the 17-ketogenic steroids were depressed in only 3 of the 4 patients studied. All major 17-ketosteroids were decreased; 11β-hydroxyandrosterone was least affected.

The present study was undertaken to evaluate the effect of Nilevar on adrenocortical function employing as indices levels of plasma 17-hydroxycorticosteroids (17-OHCS) and their response to ACTH.

Materials and methods. Five patients, 4 males and one female, were studied. Their ages and diagnoses are presented in the table. All were treated over a prolonged period with 17α-ethyl-19-nortestosterone, 10 mg 3 times a day orally. Plasma 17-hydroxycorticosteroids (17-OHCS) were determined using the method of Silber and Porter as modified by Peterson(3). Normal control levels range from 4 to 30 μg/100 ml of plasma in our laboratory. The adrenocortical response to ACTH was measured by the effect on plasma 17-OHCS levels of 25 USP units of ACTH in 500 ml of 0.9% saline in water given intravenously over 6 hours. Control samples were obtained and the infusions begun between 8 and 9 a. m. The anabolic effectiveness of Nilevar was evaluated by weight gain, increase of appetite, muscle mass and strength as well as loss of bone pain.

Results. All patients had a beneficial effect from the Nilevar. E. K. had a 15-lb weight gain. K. L. had marked reduction in bone pain and the other 3 patients showed significant increases in appetite, muscle mass and strength.

The levels of plasma 17-OHCS and their rise after ACTH, prior to and during administration of Nilevar, are shown in the Table. The control plasma 17-OHCS levels were all within normal limits and were unaffected by Nilevar, 30 mg daily, administered for 12 to 80 days. In 4 of the 5 patients, the response

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TABLE I. Effect of 17 α-Ethyl-19-Nortestosterone (Nilevar) on Plasma 17-Hydroxycorticosteroids and on Their Response to ACTH.

Patient	Sex	Age	Diagnosis	Pre-Nilevar, plasma 17-OHCS, μg/100 ml		Nilevar, 30 mg/day p.o. (No. of days)	Post-Nilevar, plasma 17-OHCS, μg/100 ml	
				Pre-ACTH	Post-ACTH		Pre-ACTH	Post-ACTH
A.S.	♂	59	Diabetes mellitus, hip fracture, osteomyelitis	17.9 30.3	56.2	14 50	24.9 33.4	68.1
H.M.	♂	51	Hip fracture, osteomyelitis	28.5 13.4	41.0	14 50	16.3 13.9	42.4
F.G.	♂	60	Osteoporosis, peripheral neuritis	23.2 17.6	72.4	12 80	19.2 16.6	77.6
E.K.	♂	23	Regional ileitis, cachexia	23.0	50.3	40	16.2	35.2
K.L.	♀	58	Rheumatoid arthritis, osteoporosis	17.3	21.9	40	26.6	43.8

of the plasma 17-OHCS to ACTH was not altered by Nilevar therapy for 40 to 80 days. K.L. had only a slight rise in the plasma 17-OHCS prior to therapy and a normal response after 40 days of Nilevar. In patient F.G., the control levels of plasma 17-OHCS were normal prior to and after therapy; however, the levels of 17-OHCS were elevated after ACTH. This increased response of the plasma 17-OHCS levels was unaltered by Nilevar therapy for 80 days.

It is well known that cortisone and its derivatives will suppress the adrenal cortex and its ability to respond to ACTH, as measured by the plasma 17-OHCS, and will produce adrenal atrophy. On the other hand, testosterone propionate and 17α-methyl-19-nortestosterone (unpublished) do not alter control plasma 17-OHCS levels. Similarly, these studies indicate that adrenocortical function, as evaluated by plasma 17-OHCS levels and response to ACTH, was unaltered by Nilevar given orally over a prolonged period of time.

Conclusions. In 5 subjects receiving clinically effective oral doses of Nilevar, there was no alteration in adrenocortical function as measured by plasma 17-OHCS levels before and after intravenous ACTH.

1. Brooks, R. V., *J. Endocrinol.*, 1956, v13, 34.
2. Brooks, R. V., Prunty, F. T. G., *J. Endocrinol.*, 1957, v15, 385.
3. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brody, B. B., Bunim, J. J., *J. Clin. Invest.*, 1955, v34, 1779.

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Studies on Copper Metabolism. XXVI. Plasma Copper in Patients with Tropical Sprue.* (24117)

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Although increased concentration of copper in plasma has been observed in a wide variety of clinical disorders(1), hypocupremia has been observed in relatively few conditions. It has been observed consistently in newborn infants(2); it is the usual finding in hepatolenticular degeneration (Wilson's disease)(3); and it has been observed frequently in the nephrotic syndrome(4). In addition, hypocupremia has been recorded in an occasional patient with hypochromic anemia(5,6, 7), idiopathic hypoproteinemia(8,9), kwashiorkor(6), and celiac disease(6,10). The causes of hypocupremia were discussed recently and a classification presented(7).

Because of the finding of hypocupremia in 2 patients with "non-tropical" sprue and severe microcytic hypochromic anemia(9), a study of plasma copper in patients with tropical sprue and megaloblastic anemia was undertaken. The results of this investigation are here reported. Observations on 6 patients with megaloblastic anemia of pregnancy are also presented.

Materials and methods. Blood samples were obtained from 29 Puerto Rican subjects with sprue in relapse. The diagnosis of sprue was based on clinical findings of chronic diarrhea, flatulence, weight loss, weakness and glossitis; macrocytic anemia with typical megaloblastic morphology in bone marrow; and laboratory evidence of intestinal malabsorption. The average hemoglobin value for the group was 5.5 g % (1.2 to 10.9 g %). Malabsorption was demonstrated for at least 2 test substances, usually xylose, Vit. A, or butter(11). In addition, steatorrhea was demonstrated by a modified fat balance technic in all 21 subjects on whom such studies were

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TABLE I. Summary of Data.

Condition	Plasma copper, μg % No. subjects	Mean ± S.D. (range)	α_2 globulin, % of total proteins No. subjects	Mean ± S.D. (range)
Normal	228	116 ± 14 (68–161)	19	11.8 ± 3.5
Sprue, relapse	29	87 ± 33 (19–157)	29	6.8 ± 2.0
" , remission	27	114 ± 33 (24–186)	14	10.0 ± 3.3
Megaloblastic anemia of pregnancy	6	159 (105–242)	3	10.6 (6.5–16.0)

done(12). Blood samples were also obtained from 27 patients during remission of their illness following at least 3 months of therapy with folic acid or Vit. B_{12}. These subjects were no longer anemic but had varying degrees of persistent malabsorption, attributable presumably either to severity or duration of previous digestive tract disease. Observations were made on specimens of plasma from 6 patients with megaloblastic anemia of pregnancy. In all 6 patients the hemoglobin was less than 9.5 g% and megaloblastic changes were present in the bone marrow. Malabsorption and steatorrhea were not present. Plasma copper was determined by the method of Gubler, *et al.*(13). Values in normal subjects have been reported(4,14). Filter paper electrophoresis of serum proteins was performed on specimens from normal subjects and patients with sprue. Electrophoretic components were estimated by means of a Spinco "Analytrol" instrument. Total serum protein measurements were not done.

Results. The mean plasma copper concentration (± 1 S.D.) of the 29 patients with untreated sprue was 87 ± 33 μg %, as compared with the normal mean of 116 ± 14 μg % (Table I). The difference between the means of the 2 groups is highly significant ($t = 4.5$; $P = <0.001$). Values in 18 (62%) of patients were less than 88 μg %, the lower limit of normal (–2 S.D.). Values greater than 144 μg % were observed in only 3 (10%) patients, 2 of whom were postpartum.

No correlation was observed between copper values and clinical severity or duration of the disease or severity of the anemia. Values were normal in a number of apparently severe and long standing cases of sprue, while in several clinically mild cases values were low. There was no significant difference between the sexes. Five of the 12 females were postpartum by 2 months or less. In 3 plasma copper was low (75, 87 and 87 μg %) while in 2 the values were slightly elevated.

Mean plasma copper concentration of the 27 patients with treated sprue was normal (114 ± 33 μg %). Values were less than 88 μg % in 2 of the patients (24 and 63 μg %), and in 2 others the values were above the upper limit of normal (156 and 186 μg %).

Plasma copper concentration was normal in 3 of the patients with megaloblastic anemia of pregnancy and was increased in the remaining 3.

Four patients with sprue were given 1 mg of copper, as copper acetate, intravenously daily for 10 days. One patient (B) had received no therapy prior to copper; one (C) was given a single oral dose of 5 mg of folic acid 7 days before; one (A) was given 15 mg of folic acid 10 days before; and one (D) had received 5 mg of folic acid daily for 5 months but had failed to respond hematologically to this medication.

In none of the patients was a hematologic response observed following administration of copper (Table II). In 2 of the patients copper therapy failed to increase concentration of copper in the plasma. In one (C) there was a modest increase. In the fourth patient (D) administration of copper was followed by

TABLE II. Response to Administration of Copper.

	Before copper			After copper		
Patient	Hb, g %	Retics., %	Plasma copper, μg %	Hb, g %	Retics., max %	Plasma copper, μg %
A	6	1	87	6	4	81
B	4	1	85	4	1	85
C	9	5	59	8	2	80
D	10		57	8		122

prompt and sustained increase in concentration of plasma copper to normal.

There was a significant reduction in the α_2 globulin fraction of plasma proteins of patients with untreated sprue as compared with that of normal subjects ($t = 57$; $P = <0.001$) or of treated patients ($t = 3.6$; $P = <0.001$). Simultaneous determinations of plasma copper and α_2 globulin were made in 40 patients. A significant degree of correlation between plasma copper concentration and α_2 globulin level was not observed ($r = +0.2$; $P = <0.2$).

Discussion. The present observations indicate that hypocupremia occurs in patients with tropical sprue and macrocytic anemia as well as in patients with non-tropical sprue accompanied by hypochromic anemia, as reported previously(9). The occurrence of hypocupremia in 62% of patients with untreated tropical sprue is in contrast to findings in 23 patients with pernicious anemia in relapse(1, 15). In this group, plasma copper values less than 88 μg % were observed in only 2 patients.

Three possible mechanisms may be invoked to explain the hypocupremia in sprue: (1) a reduced dietary intake of copper; (2) reduced absorption of copper by the intestine; or (3) impaired ceruloplasmin synthesis as a result of protein deficiency(7).

The first of these possible explanations seems unlikely since the daily dietary copper requirement of the adult is low and copper is abundantly supplied in natural foods(16). Copper deficiency due to a reduced dietary intake of copper is extremely rare in human subjects(7,16). Furthermore, if the hypocupremia in sprue were secondary to either a dietary deficiency or malabsorption of copper, it would be expected that parenteral administration of copper would be followed by a rapid return of the plasma copper concentration to normal. This did not occur in 3 of the 4 patients given copper.

About 95% of the copper in normal plasma is present in the form of ceruloplasmin, an α_2 globulin. In protein-deficient animals(17) and in human subjects under certain conditions(7), hypocupremia may result from inability to synthesize the protein portion (apoceruloplasmin) of the ceruloplasmin molecule. Since malabsorption of proteins, hypoproteinemia and reduction in the proportion of α_2 globulin in the plasma are all features observed in patients with sprue, it would seem reasonable to assume that in those patients in whom no rise in plasma copper followed administration of copper, the hypocupremia was secondary to deficiency of plasma protein, presumably due to impaired protein synthesis. Failure to observe a correlation between per cent of plasma α_2 globulin and plasma copper can probably be attributed to our failure to measure total serum protein concentration, thus making it impossible to calculate concentration of α_2 globulins in g %. In many conditions in human subjects, a high degree of correlation between concentration of α_2 globulin and plasma copper has been observed(18).

In one of the 4 patients given copper intravenously, a prompt, substantial and sustained increase in plasma copper occurred following intravenous administration of copper. In this patient, α_2 globulins constituted 11.6% of the total proteins, a normal value. Therefore, in this patient, malabsorption of copper may have been the explanation for the hypocupremia.

The observation of hypercupremia in 3 patients with megaloblastic anemia of pregnancy is in keeping with the hypercupremia which accompanies normal pregnancy(2). Four of the 6 patients with megaloblastic anemia of pregnancy were studied only in the early post-partum period. Plasma copper values in 3 of these were within normal limits. It was shown previously that the hypercupremia of pregnancy decreases rapidly during the post-partum period(2).

Summary. 1. Plasma copper was determined in 29 patients with sprue in relapse, 27 patients with sprue in remission, and 6 patients with megaloblastic anemia of pregnancy. 2. Mean plasma copper concentration (± 1 S.D.) in patients with sprue in relapse was 87 ± 33 μg %; in treated patients, 114 ± 33; and in patients with megaloblastic anemia of pregnancy, 159 (105 – 242). 3. It is suggested that hypocupremia in sprue may frequently be due primarily to an inability to

synthesize the protein portion of ceruloplasmin. In a few patients malabsorption of copper may be a contributing factor.

1. Lahey, M. E., Gubler, C. J., Cartwright, G. E., Wintrobe, M. M., *J. Clin. Invest.*, 1953, v32, 329.

2. Fay, J., Cartwright, G. E., Wintrobe, M. M., *ibid.*, 1949, v28, 487.

3. Bearn, A. C., *Am. J. Med.*, 1957, v22, 747.

4. Cartwright, G. E., Gubler, C. J., Wintrobe, M. M., *J. Clin. Invest.*, 1954, v33, 685.

5. Sturgeon, P., Brubaker, C., *A.M.A. J. Dis. Child.*, 1956, v92, 254.

6. Lahey, M. E., *Am. J. Clin. Nutrition*, 1957, v5, 516.

7. Zipursky, A., Dempsey, H., Markowitz, H., Cartwright, G. E., Wintrobe, M. M., *A.M.A., J. Dis. Child.*, 1958, in press.

8. Ulstrom, R. A., Smith, N. J., Heimlich, E. M., *ibid.*, 1956, v92, 219.

9. Cartwright, G. E., *Am. J. Clin. Nutrition*, 1955, v3, 11.

10. Axtrup, S., *The Blood Copper in Anaemias of Children with Special Reference to Premature Cases*, Lund, Sweden, P. H. Lundstedt Univ.-Bokhandel, 1946.

11. Gardner, F. H., Perez-Santiago, E., *A.M.A. Arch. Int. Med.*, 1956, v98, 467.

12. Perez-Santiago, E., Butterworth, C. E., Jr., *Am. J. Dig. Dis.*, 1957, v2, 225.

13. Gubler, C. J., Lahey, M. E., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., *J. Biol. Chem.*, 1952, v196, 209.

14. Lahey, M. E., Gubler, C. J., Cartwright, G. E., Wintrobe, M. M., *J. Clin. Invest.*, 1953, v32, 322.

15. Cartwright, G. E., Huguley, C. M., Jr., Ashenbrucker, H., Fay, J., Wintrobe, M. M., *Blood*, 1948, v3, 501.

16. Cartwright, G. E., Copper Metabolism: A Symposium on Animal, Plant and Soil Relationships, edited by W. D. McElroy and B. Glass, Johns Hopkins Univ. Press, Baltimore, 1950, 274.

17. Cartwright, G. E., Wintrobe, M. M., *J. Biol. Chem.*, 1948, v176, 571.

18. Lahey, M. E., Gubler, C. J., Brown, D. M., Smith, E. L., Jager, B. V., Cartwright, G. E., Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1953, v41, 829.

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Observations on Blood Flow During Spontaneously Occurring Traube-Hering Waves. (24118)

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Rhythmic fluctuations in blood pressure which are slower than the respiratory rate and on which respiratory and cardiac waves are superimposed are commonly designated by the names of several or all of the authors describing them in the latter part of the last century; Traube(1), Hering(2), and Mayer(3). These investigators agreed that the waves in blood pressure were probably due to rhythmic changes in peripheral resistance secondary to rhythmic impulses conveyed from the central nervous system *via* the sympathetic nerves. Hering, further, felt on the basis of his studies that the respiratory center was the source of the rhythmic impulses which acted on the vasomotor center by way of its connections with the latter. More recently evidence has been reported indicating that chemo- and baro-receptors may be the ultimate starting point of the rhythmic impulses(6,7). Much evidence has since accumulated to support the view that rhythmic alterations in peripheral resistance account for blood pressure fluctuations. Thus it has been shown that the diameter of arteries in the ear of rabbits and dogs fluctuate rhythmically in time but opposite in direction to the rhythmic changes in arterial pressure(4). Blood flow as estimated by the hot-wire anemometer technic fluctuates similarly when measured in the leg muscles of the cat(5). Recent studies in humans using plethysmograph technics have shown similar findings and, moreover, indicate that the Traube-Hering waves are commonly seen in normal, unanesthetized subjects(8-11). Direct measurements of blood flow during the

occurrence of Traube-Hering waves seem to be almost entirely lacking in the literature, only one report being found in publications of the past 20 years. This communication (12) concerned the finding of rhythmic changes in blood flow to the hind limb of a dog, the flow changes being synchronous with but in the opposite direction to the variations in systemic artery pressure. The present communication reports briefly 2 additional instances in which direct measurements of blood flow were made during spontaneous occurrence of Traube-Hering waves in the dog.

Material and methods. Blood flow and blood pressure recordings were made in 2 separate groups of experiments on 6 dogs each. In one group the blood flow in the saphenous artery (a small branch of the femoral artery supplying the skin mainly) was measured by the differential pressure technic employing a differential strain gauge to record pressures above and below a constricted area in a plastic tube the distal end of which was inserted into the distal end of the saphenous artery near its origin. Blood was withdrawn from the opposite femoral artery and pumped through this tubing into the saphenous artery at a pressure determined by a Starling resistor which allowed shunting of blood above a fixed pressure head. Pulsatile pressure approximating normal values was employed. Thus the flow of blood through the vascular bed under study was the flow under a controlled pressure and variations in flow are inversely related to the vascular resistance. In the second group of experiments blood flow into the distal vertebral arteries was measured with electrically recording rotameters which were incorporated into the arterial side of a DeWall-Lillehei pump oxygenator, the venous return being obtained from the femoral veins. The perfusion pressure was controlled as in the first series. The carotid arteries were ligated and the soft tissues of neck tightly compressed with a metal band save for the trachea, the external jugular veins, the carotid arteries and the vagosympathetic nerve trunks. Anesthesia was induced with chloralose, 100 mg/kg, intravenously. The femoral artery blood pressure was recorded *via* a Statham strain gauge on a Sanborn polyviso recorder along with the blood flow.

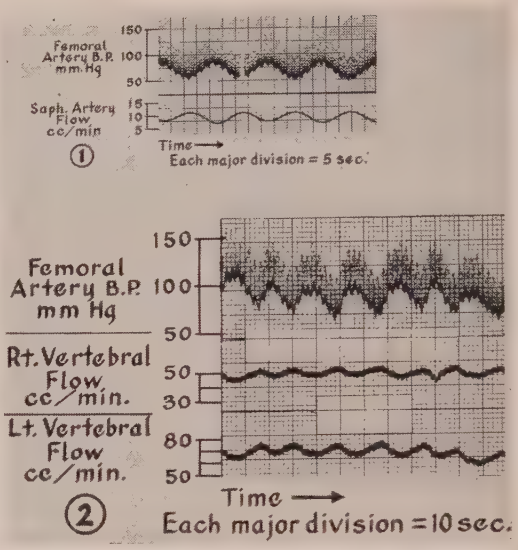

FIG. 1 and 2. Relationship between blood flow and femoral artery pressure observed in 2 instances in which Traube-Hering waves spontaneously occurred during an experiment.

Results. One of 6 animals in each of the 2 experimental groups spontaneously developed slow rhythmic fluctuations in blood pressure which had a period of about 18 to 20 seconds. Both animals were in good general condition when this occurred and remained so for a considerable period of time thereafter. The variations in blood flow under independently controlled pressure (Fig. 1,2) are seen to be exactly synchronous in time but opposite in direction to the fluctuations in systemic blood pressure. This finding indicates that in the absence of changes in perfusion pressure there is an increase in regional vascular bed resistance synchronous with the systemic blood pressure rise. This confirms the conventional view that the Traube-Hering waves are due at least in large part to centrally mediated changes in vasomotor tone. This report is being made primarily because in previous studies which have come to our attention with one exception the perfusion pressures in regional beds under study had not been controlled.

Summary. Two experiments are reported in which regional blood flow under controlled perfusion pressure was being measured by direct means along with systemic blood pressure and in which Traube-Hering waves were

seen to occur. Rhythmic changes in the regional blood flow were observed which were synchronous with but opposite in direction to the changes in systemic blood pressure. This agrees with the conventional view that changes in blood pressure in this situation are secondary to changes in peripheral resistance.

1. Traube, L., *Centralbl. f. d. Med. Wiss.,* 1865, v56, 881.
2. Hering, E., *Sitzber. d. Kaiserl. Akad. Wiss., Wien.,* 1870, v60, 829.
3. Mayer, S., *ibid.,* 1876, v74, 281.
4. Wilson, H. C., *Am. J. Physiol.,* 1936, v116, 295.
5. Irving, L., *Am. J. M. Sci.,* 1938, v196, 988.
6. Andersson, B., Kenny, R. A., Neil, E., *Acta Physiol. Scand.,* 1950, v20, 203.
7. Guyton, A. C., Harris, J. W., *Am. J. Physiol.,* 1951, v165, 158.
8. Burton, A. C., *ibid.,* 1939, v127, 437.
9. Matthes, K., Malikiosis, X., *Z. Exp. Med.,* 1943, v112, 476.
10. Cerletti, A., *Helv. Physiol. Acta,* 1945, v3, 537.
11. Steinmann, B., Rickenbach, K., Gianoli, A., *Cardiologia,* 1953, v23, 154.
12. Green, H. D., Lewis, R. N., Nickerson, N. D., PROC. SOC. EXP. BIOL. AND MED., 1943, v53, 228.

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Effect of Dicumarol on Bleeding Time.* (24119)

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Roskam(1) reported that heparin has only a slight effect on bleeding time. This observation has been confirmed many times clinically. It is also evident from simple clinical observation that the newer indirect anticoagulants such as dicumarol have little effect on normal bleeding. However, there has not been any recent investigation of the effect of dicumarol on bleeding time. Roskam showed that the effect of heparin on bleeding time depended on technical details of method used. In our study, we used the original method of Roskam and Pauwen(2). Link(3) reported that a certain number of normal rabbits fail to show increase in prothrombin time with dicumarol (non-reactors). This provides a useful control in many experiments on dicumarol (4). As reported separately(5), administration of ACTH to rabbits results in rabbits previously refractory to dicumarol showing elevated prothrombin times with the drug. One series of our rabbits were therefore treated with ACTH in addition to dicumarol.

Methods. Bleeding times were determined on ear of rabbit by the method of Roskam and Pauwen(2). The ear was shaved, the rabbit immobilized. Ten cuts were made on each ear and the ears bathed with distilled water at 30°C and pH 7.0. Time for bleeding to stop from each cut was recorded and mean bleeding time with standard deviation calculated. Prothrombin times were done by the usual Quick procedure using rabbit brain thromboplastin. Dicumarol was given orally as a single dose of 5 mg/kg. ACTH was given to some rabbits as a single intramuscular dose of 5 mg/kg at the same time as dicumarol.

Results. Eighteen rabbits were given dicumarol and prothrombin times and bleeding times determined before (day 0) and on the third day. Five rabbits were similarly treated and prothrombin times and bleeding times determined before and on the fifth day. Eleven rabbits received both dicumarol and ACTH and prothrombin times and bleeding times determined before and on fifth day. The results are shown in Table I. P values are for value of mean bleeding time compared to the same value on day 0. Comparing bleeding time on third day with that at beginning of experiment, the average of mean bleeding times for the 18 rabbits showed a slight but significant increase. Eight of the 18 rabbits gave a sig-

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TABLE I. Effect of Dicumarol on Prothrombin Time and Bleeding Time.

Dicumarol alone						Dicumarol alone					Dicumarol and ACTH					
Prothrombin time (sec.)		Mean bleeding time (sec.)			Day of	Prothrombin time (sec.)		Mean bleeding time (sec.)			Prothrombin time (sec.)		Mean bleeding time (sec.)			Day of
Day 0	Day 3	Day 0	Day 3	P	death	Day 0	Day 5	Day 0	Day 5	P	Day 0	Day 3	Day 0	Day 3	P	death
11.2	>480	136 ± 7.2	107 ± 13.3	<.1	4	8.3	11.0	170 ± 11.2	151 ± 44.7	>.5	23.6	66.5	135 ± 7.9	*199* ± 31.6	<.01	7
12.4	"	129 ± 4.4	*193* ± 15.1	<.01		8.6	8.3	193 ± 21.2	182 ± 25.9	"	9.5	44.2	184 ± 10.8	*274* ± 35.9	<.05	6
10.2	219	136 ± 13.6	146 ± 21.6	<.5		10.0	10.2	170 ± 17.0	155 ± 25.3	"	25.8	276	146 ± 7.0	177 ± 19.0	<.2	
9.8	>480	126 ± 12.9	154 ± 26.5	<.4	4	8.6	9.5	178 ± 17.1	161 ± 23.0	"	14.8	>480	212 ± 28.4	145 ± 16.5	<.1	
9.3	"	135 ± 8.4	129 ± 7.2	<.5	"	11.0	9.7	170 ± 20.1	183 ± 39.2	"	10.1	61.8	180 ± 31.2	192 ± 31.5	>.5	
11.2	234	128 ± 8.8	*163* ± 12.5	<.05	"						10.8	50.2	198 ± 22.0	181 ± 16.8	"	
8.6	13.3	134 ± 9.6	*223* ± 29.5	<.02			M:	176 ±	166 ±	"	9.7	43.1	193 ± 10.8	161 ± 15.7	"	
9.3	309	126 ± 21.8	196 ± 27.3	<.1							9.6	314	151 ± 15.9	188 ± 5.8	<.1	
10.1	51.2	155 ± 30.7	119 ± 8.4	<.3	4						10.1	33.1	147 ± 12.7	174 ± 14.0	<.2	6
15.2	348	142 ± 11.1	156 ± 21.7	<.5.							27.3	238	164 ± 20.9	*296* ± 22.9	<.01	
9.5	323	120 ± 3.9	*204* ± 9.7	<.01	12						8.9	>480	206 ± 19.7	152 ± 6.3	<.05	
9.9	17.2	148 ± 19.2	*220* ± 12.6	<.01												
10.6	22.8	126 ± 17.7	168 ± 25.0	<.2								M:	170 ± 8.4	194 ± 19.4	<.2	
8.4	97.2	124 ± 10.4	*262* ± 45.2	<.02	4											
10.2	>480	126 ± 14.9	107 ± 8.9	.3	"											
26.4	"	156 ± 8.8	*225* ± 21.2	.01	6											
10.6	15.8	156 ± 4.7	*218* ± 23.2	<.05												
9.6	17.4	135 ± 12.6	173 ± 22.8	.3												
	M:	135 ± 4.2	176 ± 10.6	<.05												

Mean values showing significant difference from those for day 0 are italicized.

nificantly greater bleeding time on third day. Three of the 4 non-reactor rabbits which showed only a slight increase in prothrombin time increased in bleeding time. On the other hand, 9 rabbits showed no increase in bleeding time, and 7 of these had quite prolonged prothrombin times. Six rabbits showed an increase in both values. One animal with increased prothrombin time showed a significant decrease in bleeding time. There was therefore little relationship between the 2 values.

There have been reports of dicumarol affecting vessels directly(6). The mean bleeding time and prothrombin time were determined for 5 non-reactor rabbits on fifth day after receiving dicumarol. There was no change in bleeding time. As found previously, when dicumarol and ACTH were administered simultaneously to rabbits, all animals showed increase in prothrombin time. Three of these rabbits gave an increased bleeding time 3 days after receiving dicumarol and ACTH. This incidence was no greater than with dicumarol alone and the other rabbits were unaffected. One animal showed a significant decrease in bleeding time. On the other hand one rabbit died of hemorrhage without increase in bleeding time from ACTH and dicumarol.

A most remarkable finding was that the animals on which bleeding time determinations were performed on third day and in all of which the bleeding had stopped by 5 minutes after the cut, showed bleeding commencing again 1-2 hr later. Eight of these animals were dead the next morning in their cages and 4 died in the next 3 days. Some small amounts of blood were present in the cage and on the fur. When the animals were autopsied all organs were found to be exceedingly pale and anemic. Lung pathology was observed in 4 rabbits. The general picture was of extensive hemorrhage and shock although large amounts of blood were not found in the cage. The picture was identical with that already observed by Jaques *et al.*(7,8) for rabbits on anticoagulants subjected to stress. It appears to be due to a non-specific hemorrhage or leaking of blood into tissues all over the body without any particular area of specific hemorrhage. It is particularly remarkable that rabbits which had a perfectly normal bleeding time at 2 p. m. were dead from hemorrhage the next morning.

Discussion. That animals bleed to death internally following determination of bleeding time externally, even though bleeding time is normal, is a remarkable observation. It can be explained on the basis of previous observations of Jaques *et al.* that when rabbits on dicumarol are subjected to stress, death from hemorrhage occurs in a high percentage of animals. Determination of bleeding time by repeated cutting of the rabbit ear seems to be an effective stress stimulus in the rabbit as judged by this high mortality. There is no increase in mortality when ACTH is added. As reported separately(5), administration of ACTH instead of stress to dicumarolized rabbits also results in hemorrhagic death in rabbits. Bounameaux, van Cauwenberge and Roskam(9) demonstrated that the immediate effect of ACTH and cortisone was to shorten bleeding time of rabbits. This is also illustrated by the fact that there was no increase in bleeding time with dicumarol plus ACTH and indicates that bleeding time is not a measure of the changes in vessels due to ACTH.

Summary. Rabbits were treated with dicumarol and dicumarol plus ACTH and prothrombin times and bleeding times determined. A slight increase in bleeding time was observed after 3 days on dicumarol, in 50% of the rabbits. Half the rabbits died in the next 24-72 hr from generalized non-specific hemorrhage. This was not related to the previous increase in bleeding time. Bleeding did recur and continue from the cut ear but hemorrhage was much more extensive than this. It is suggested that determination of bleeding time is a marked stress procedure in rabbits.

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1. Roskam, J., *Arch. Int. Pharmacodyn.*, 1942, v68, 66.

2. Roskam, J., Pauwen, L., *ibid.*, 1937, v57, 450.

3. Link, K. P., *Harvey Lecture,* 1943-4, v39, 162.

4. Jaques, L. B., Froese, E. Lepp, O'Toole, R. E., Spinks, J. W. T., *Arch. intl. de pharmacol.*, 1957, III, v4, 478.

5. van Cauwenberge, H., Jaques, L. B., *Can. Med. Assn. J.*, in press.
6. Dale, D. U., Jaques, L. B., *ibid.*, 1942, v46, 546.
7. Lepp, E., Chubaty, W., Jaques, L. B., *Can. J. Med. Sc.*, 1953, v31, 173.
8. Jaques, L. B., Mogenson, G. J., Fisher, L. M., *Trans. Roy. Soc. Can. Third series*, 1956, v50, Sec. V, 9.
9. Bounameaux, Y., van Cauwenberge, H., Roskam, J., *Schweiz. Med. Wchschr.*, 1954, v84, 789.

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Purification of Erythropoietin by Ion-Exchange Chromatography.* (24120)

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We reported(1,2,3) that erythropoietic activity of acidified, boiled plasma filtrates prepared from phenylhydrazine anemic rabbits was associated with a protein having paper electrophoretic mobility of an alpha globulin and chemical characteristics of an acid glycoprotein. Small molecular weight, acidic glycoproteins are found in Cohn Fraction VI(4). In preliminary work with plasma from phenylhydrazine anemic rabbits and from a patient with polycythemia vera we found erythropoietic activity in Fraction VI (unpublished). The present study was therefore designed to isolate, by ion-exchange chromatography, acidic glycoproteins from the crude source of boiled anemic rabbit plasma.

Materials and methods. Acidified, boiled plasma filtrate (APF) obtained from phenylhydrazine anemic, adult, white male rabbits was prepared by modification of method of Gordon(5) as previously described(3). The filtrate was dialyzed in the cold 48 hours against repeated changes of distilled water and then lyophilized. One liter of plasma yielded 500 to 600 mg of lyophilizate. The lyophilizate, which possesses erythropoietic activity by our previously described method (3), was fractionated on diethylaminoethyl (DEAE) cellulose ion-exchange columns(6). Two separation procedures (I,II) were utilized. Procedure II was designed for a more definitive fractionation on the basis of results obtained with procedure I. The separation experiments are summarized in the elution diagrams in Fig. 1 and 2. Fraction pools as designated in Fig. 1 were dialyzed, lyophilized and redissolved in 0.9% NaCl. Along with unmodified APF starting material, and control saline injections, these fractions were assayed for erythropoietic activity. Fraction pools as designated in Fig. 2 were assayed directly, using a solution of 0.9% NaCl in 0.01 M sodium acetate buffer as a control injection. Female, Sprague-Dawley rats (average weight, 220 g) were injected subcutaneously daily for 4 days with 2 mg by dry weight (in 2 ml 0.9% NaCl) of the respective fractions from procedure I. The fractions from procedure II were injected on a similar schedule, with a daily dose of 0.5 mg by protein content. Protein content was estimated on the effluent fraction pools by the ratio of optical density measurements at 280 and 260 mμ according to the method of Warburg and Christian(7). On day 4, the animals were given an intravenous injection of 0.5 μc iron-59 as iron citrate contained in 0.25 ml of solution. The per cent of injected dose of iron-59 appearing in the total red cell mass in 24 hours was estimated by standard scintillation counting technics on whole blood obtained by aortic exsanguination under ether anesthesia. The total red cell mass was calculated from the microhematocrit assuming an average blood volume of 5.18 ml/100 g body weight(3). Prior to sacrifice, blood from tail vein was obtained for reticulocyte enumeration by the dry cresyl-blue method.

Results. Erythropoietic activity of isolated

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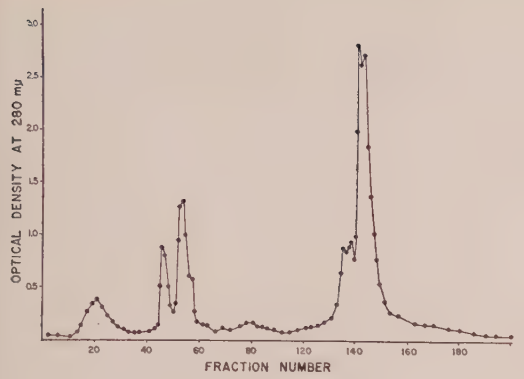

FIG. 1. Effluent diagram, procedure I. 500 mg APF dissolved in 30 ml 0.01 M sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (40 × 2.5 cm) which had been partially equilibrated to pH 6.1 with 0.01 M acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1-77. Gradient started at fraction 77 by continuous introduction of 0.01 M acetate buffer, pH 4.8 plus 0.25 M NaCl into a constant volume reservoir of 200 ml 0.01 M acetate buffer. Column operated at constant temperature of 25°C. Flow rate 0.65 ml/min. and fraction vol 6.5 ml. Fraction pool 44-56 (Fraction A), 132-138 (Fraction B), 142-150 (Fraction C).

fractions is presented in Tables I and II. Fraction A, washed from column in the solvent buffer, and Fraction B showed no erythropoietic activity. The small peak appearing in the elution diagram before Fraction A was also inactive, but was tested in only 2 animals. Fraction C contained the majority of erythropoietic activity and was more potent than the starting material. In 0.15 M NaCl the peak of Fraction C was homogeneous in the ultracentrifuge. The centrifuge data suggested a molecular weight of about 10,000. By free electrophoresis, barbiturate buffer pH 8.6, ionic strength 0.1, this peak yielded 3 components with mobilities (μ x 10^5) of –6.31, –5.23 and –4.49. These mobilities correspond to the mobilities of the alpha globulins.

In procedure II erythropoietic activity was almost entirely confined to Fraction G. The minimal response induced by Fraction F suggests that it is contaminated by a small amount of the active fraction. From the character of the effluent diagram, it appears that the material in Fraction G is homogeneous. On paper electrophoresis in veronal buffer pH 8.6, ionic strength 0.075, it migrates as a single component with a mobility between that of alpha-2 and alpha-1 globulin. It stains as a glycoprotein. From its behavior on the DEAE-cellulose column it has a low isoelectric point. Its small molecular size is indicated by the failure to sediment activity when APF is centrifuged at 103,000 g for 24 hours. Its protein content as estimated by the method of Warburg and Christian is 69.3%. Its sialic acid content as determined by Winzler's modification of the method of Ayala, *et al.*(9) is 15.6%. Its hexose content by the anthrone reaction is 7.7% as glucose equivalent, and its glucosamine content by modification of the method of Elson and Morgan(9) is 10%. Preliminary studies suggest that the glucose content is low, and that at least a part of the hexose is present as galactose. The sialic acid content of the material is relatively high when compared with that reported for other serum glycoproteins(8),

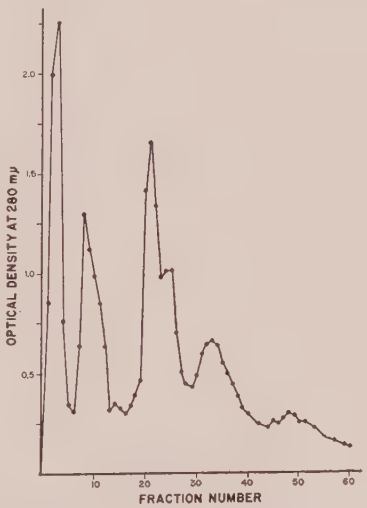

FIG. 2. Effluent diagram, procedure II. 300 mg APF dissolved in 20 ml 0.01 M sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (30 × 1 cm) which had been partially equilibrated to pH 6.0 with 0.01 M acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1-13. Gradient started at fraction 13 by continuous introduction of 0.01 M acetate buffer, pH 4.8 plus 0.25 M NaCl into a constant volume reservoir of 125 ml 0.01 M acetate buffer. Column operated at constant temperature of 5°C. Flow rate 0.08 ml/min. Fraction vol: fractions 1-13, 6.5 ml; fractions 14-40, 3.5 ml. Fraction pool 1-4 (Fraction D), 7-12 (Fraction E), 19-26 (Fraction F), 30-40 (Fraction G).

and is probably responsible for its low isoelectric point. Fraction F contains 9% sialic acid, while Fractions D and E contain 7%.

Fraction G was obtained in a yield of 16 mg. Calculations based on amount of starting material indicate that the active fraction stimulates erythropoiesis when injected in 50 μg quantities. Preliminary dose response data using Fraction G suggest that the injection of 10 μg per day will induce a measurable erythropoietic response.

This separation and isolation of erythropoietin is reproducible, and similar results have been obtained from different lots of starting APF material. Studies are currently underway to further characterize this erythropoietic factor.

Summary. A method for purification of erythropoietin from the filtrate of acidified, boiled plasma prepared from phenylhydrazine anemic rabbits utilizing DEAE-cellulose ion-exchange columns has been described. The active erythropoietic factor thus prepared has been partially characterized and shown to be a low molecular weight, acidic glycoprotein.

TABLE I. Erythropoietic Activity of Effluent Fractions, Procedure I.

Fraction pool	Reticulocytes (%)*			Fe^{59} incorporation in RBC (%)*		
A	2.1	.4	(4)	26.7	4.3	(7)
B	1.6	.5	(4)	27.4	10.4	(4)
C	*6.1*	*1.5*	(4)	*50.5*	*2.2*	(9)
APF	*3.4*	*.6*	(4)	*39.9*	*4.4*	(4)
Saline control	1.8	.3	(4)	21.0	3.6	(7)

* Mean ± S.D.

Figures in parentheses indicate No. of animals in group.

Italicized values statistically significant, P = .01 or less.

TABLE II. Erythropoietic Activity of Effluent Fractions, Procedure II. (Four animals/group.)

Fraction pool	Reticulocytes (%)*		Fe^{59} incorporation in RBC (%)*	
D	2.7	.6	28.3	6.6
E	2.5	.4	26.9	4.5
F	2.6	.4	*29.6*	*1.0*
G	*6.5*	*1.3*	*47.6*	*2.5*
APF	*3.5*	*.5*	*40.7*	*6.9*
NaCl-acetate control	2.3	.4	21.0	2.6

* Mean ± S.D.

Italicized values statistically significant, P = .01 or less.

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1. Rambach, W. A., Cooper, J. A. D., Alt, H. L., *J. Lab. & Clin. Med.,* 1956, v48, 933.

2. ———, *Proc. VI Internat. Cong. Internat. Soc. Hem.,* Grune & Stratton, N. Y., p773, 1958.

3. Rambach, W. A., Alt, H. L., Cooper, J. A. D., *Blood,* 1957, v12, 1101.

4. Schmid, K., *J.A.C.S.,* 1955, v77, 742.

5. Gordon, A. S., Piliero, S. J., Kleinberg, W., Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255.

6. Peterson, E. A., Sober, H. A., *J.A.C.S.,* 1956, v78, 751.

7. Warburg, O., Christian, W., *Biochem. Z.,* 1941, v310, 384.

8. Winzler, R. J., *Methods of Biochemical Analysis,* vII, Interscience Publishers, N. Y., p299, 1955.

9. Palmer, J. W., Smyth, E. M., Meyer, K., *J.B.C.,* 1937, v119, 491.

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Method for Studying Translocation of Some Radioisotopes into Organs from Site of Injection.* (24121)

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Therapeutic application of a radioisotope is outlined by knowledge of its fate in the body. This information is acquired by testing amount of radioactivity in organs of animals

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sacrificed at requisite intervals after radioisotope injection. For pure beta emitters with only a short range of irradiation, the knowledge of their distribution is practically equivalent to knowledge of their action. Thus, therapeutic possibilities of radioactive Yttrium (Y^{90}) were investigated by Kyker(1) in rats serially sacrificed at intervals of 1, 3, 6 and 8 days after injections by various routes. Lahr, Olsen, Gleason and Tabern(2) studied on comparative lines the distribution of pure or prevalently beta emitters (Y^{90} chloride and phosphate, radioactive chromic phosphate and colloidal Au^{198}) 5 to 7 days after subcutaneous or intracavitary administration. Ruth Lewin *et al.*(3) have sacrificed ascites tumor bearing mice on third day after i.p. injection of Y^{90}. It was our purpose to provide additional information by injecting beta emitting isotopes subcutaneously into the mouse tail and by amputating this organ at various intervals between injection and autopsy. Accordingly, we were able to record level of radioactivity at site of injection at various time intervals after administration of an isotope; and, moreover, to correlate these findings with successive changes in amount of radioactivity stored in organs. The results and their interpretation are reported below.

Material and methods. a) Swiss Albino male mice of about 25 g weight were used. b) Radioactive yttrium chloride ($Y^{90}Cl_3$) was obtained from Abbott Laboratories, Oak Ridge, Tenn.; radioactive chromic phosphate ($CrP^{32}O_4$) from the same, North Chicago, Ill. Doses of 0.05 mc in 0.1 ml were used for injection. c) *Technics of injections and amputations.* Doses of 0.05 mc of $Y^{90}Cl_3$ solution and of $CrP^{32}O_4$ colloidal suspension in 0.05 or 0.1 ml were injected into ventral surface of tail of mice. At intervals of 1 minute to 3 days after injection, the tails were serially amputated and preserved until the 3rd day when every mouse was sacrificed. Their organs (liver, lung, spleen, kidney), tails, and heart blood (0.3 ml) were emulsified and tested for radioactivity content. The material was injected into a thin layer of connective tissue on ventral side of tail, carefully avoiding blood vessels. The tail was amputated with sharp surgical scissors at its junction with the sacral spine. It was refrigerated until the animal was sacrificed for autopsy. d) *Autopsies.* The tail, blood (0.3 ml of unclotted blood from heart), and the most important visceral organs (liver, spleen, both kidneys and the lung) were ground and suspended in detergent solution, placed into tin cups, and dried. Whole organs were used, since their weight was significantly constant in mice of the same batch. e) *Radioactivity counts.* Counting was performed with a Geiger tube and scaler. Specimens from all mice of the same group (sacrificed after same interval) were counted on same day within 4 to 5 hours. It is realized that the decay between the first and last count may amount to 5% for isotopes of short half life. To avoid this error we count, at the present time, all specimens within 1 hour by working with less active material. f) *Method of calculation.* To obtain strictly comparable data, we calculated the individual pattern of radioactivity for each mouse, using radioactivity in standard amount of heart blood (0.3 ml) as a unit. For each group of animals, the average pattern and extreme variations were recorded in the Tables as ratios of radioactivity in blood at various stages of distribution. This method of expressing results as count ratios instead of average activity of tissue was used in our previous publications(5). For comparison of radioactivity ratio "organ/blood" in various groups, the average blood radioactivity in group 2 (amputation after 10 minutes) was also used as standard in the other groups. The last column of each Table was calculated upon this standard.

Results. The calculated ratios of radioactivity were tabulated separately for each isotope (Tables I and II) according to source of material (various organs) and interval of time elapsing between injection of isotope and removal of injected tissue, *i.e.* tail amputation. Thus, in each Table, horizontal lines show patterns of radioisotope distribution in the body at various intervals, while vertical columns illustrate the fate of the isotope in each surveyed organ.

Both Tables present consistently a common feature, *i.e.*, absence of wide variations in radioactivity content of heart blood (last

TABLE I. Spread of Y^{90} from Site of Injection (Tail) into Blood and Organs at Various Intervals before Removal of Injected Tissue (Tail Amputation).

Group	Time between Y^{90} inj. into tail and tail amputation	Radioactivity content of inj. tissue (tail) and of visceral organs expressed as products of avg blood radioactivity in the same group					Comparison of blood radioactivity in different groups, with group 2 as 1
		Tail	Lung	Kidney	Spleen	Liver	
1	1–5 min.	82.0 (80.2-100.3)	1.0 (.9-1.2)	1.7 (1.3-2.2)	2.5 (1.1-2.7)	4.4 (1.9-6.2)	1.1 (.9-1.3)
2	10	86.5 (78.7-96.1)	1.8 (1.1-2.3)	1.3 (1.2-1.6)	2.7 (1.0-2.3)	3.9 (3.4-4.2)	1.0 (1.0-1.1)
3	15–20	86.1 (80.8-92.5)	1.7 (1.2-2.2)	1.2 (1.0-1.8)	2.6 (1.3-3.3)	3.8 (3.7-4.1)	1.1 (1.0-1.1)
4	30	53.4 (42.6-59.9)	1.0 (.9-1.2)	1.3 (1.0-1.6)	1.5 (1.2-1.9)	5.1 (3.1-5.4)	1.1 (.9-1.2)
5	45	54.1 (44.9-75.2)	1.3 (.9-1.5)	1.9 (.9-2.9)	1.3 (1.0-1.5)	4.6 (3.9-5.2)	1.0 (1.0-1.1)
6	1 hr	59.7 (55.1-72.1)	1.2 (1.0-1.5)	1.7 (.9-2.7)	1.2 (1.0-1.4)	5.9 (5.0-7.2)	1.3 (1.1-1.4)
7	3	69.8 (60.1-75.4)	1.1 (1.0-1.7)	1.9 (1.1-2.4)	1.9 (1.3-2.2)	5.3 (4.4-6.1)	1.2 (1.1-1.2)
8	24	57.9 (55.5-62.0)	1.7 (1.4-1.8)	1.7 (1.3-2.2)	1.7 (1.4-2.0)	4.9 (4.1-5.0)	1.2 (1.0-1.3)
9	48	43.5 (37.6-62.4)	1.9 (1.7-2.3)	1.6 (1.2-1.7)	1.6 (1.2-1.7)	5.2 (3.8-6.1)	1.1 (1.0-1.1)
10	72	40.8 (36.7-43.3)	2.3 (2.1-2.6)	1.9 (1.5-2.3)	1.9 (1.5-2.3)	5.1 (4.2-5.8)	1.1 (1.1-1.2)

Ratio for each tissue is avg of 15 mice in each group; range of variation is shown by minimum-maximum values in parentheses.

TABLE II. Spread of CrP^{32} from Site of Injection (Tail) into Blood and Organs at Various Intervals before Removal of Injected Tissue (Tail Amputation).

Group	Time between $CrP^{32}O_4$ inj. into tail and tail amputation	Radioactivity content of inj. tissue (tail) and of visceral organs expressed as products of avg blood radioactivity in the same group					Comparison of blood radioactivity in different groups, with group 2 as 1
		Tail	Lung	Kidney	Spleen	Liver	
1	1–5 min.	56.4 (52.2-57.1)	2.8 (1.9-3.1)	1.6 (1.2-2.1)	1.4 (1.1-1.7)	3.3 (2.2-4.1)	1.2 (1.0-1.4)
2	10	52.1 (51.4-54.3)	1.2 (.9-2.9)	1.7 (1.2-2.2)	1.2 (.8-1.3)	7.5 (5.1-8.9)	1.0 (.9-1.2)
3	15–20	62.1 (47.2-71.9)	1.6 (.9-2.2)	1.8 (1.2-1.5)	1.4 (1.2-1.9)	6.7 (5.3-7.2)	1.2 (1.0-1.2)
4	30	64.4 (62.2-71.6)	1.6 (1.1-1.3)	1.4 (.9-1.2)	1.4 (.9-2.0)	12.6 (10.2-15.6)	1.0 (1.0-1.1)
5	45	69.2 (53.3-77.5)	1.2 (1.1-1.3)	1.3 (.9-1.5)	1.3 (.9-1.5)	17.3 (16.2-18.2)	1.4 (1.2-1.6)
6	1 hr	43.9 (41.3-47.8)	1.1 (.9-1.1)	1.3 (.7-1.5)	1.1 (.9-1.3)	16.9 (15.3-17.4)	1.2 (1.1-1.3)
7	3	45.1 (39.3-47.7)	1.8 (1.0-2.1)	1.9 (1.2-2.2)	1.4 (1.1-1.8)	16.4 (15.8-16.9)	1.3 (1.2-1.4)
8	24	44.7 (38.2-47.3)	2.4 (1.7-2.5)	2.2 (1.7-2.8)	1.7 (1.5-1.9)	27.0 (25.1-29.0)	1.5 (1.1-2.0)
9	48	41.1 (37.3-46.1)	2.5 (1.8-2.2)	2.7 (1.6-3.5)	2.5 (2.1-3.5)	23.3 (21.4-24.4)	1.4 (1.1-1.7)
10	72	43.1 (41.5-46.7)	2.2 (1.8-2.6)	2.6 (2.2-3.9)	2.2 (1.8-3.2)	29.3 (28.2-30.1)	1.2 (1.0-1.3)

Ratio for each tissue is avg of 15 mice in each group; range of variation is shown by minimum-maximum values in parentheses.

column); thus, changes in ratios reflected variations of radioactivity in the organs. Both isotopes showed a common trend to be partially dislocated from the injected tissue, but for each isotope timing and amount of translocation were different: Y^{90} was accumulated in the tail during the first 20 minutes after injection in a much larger amount than $CrP^{32}O_4$, but it was found to be less after 30 minutes and remained at the same level for 24 hours and then dropped again. For $CrP^{32}O_4$ the initial storage was more limited and after a transient relative increase (15 to 45 minutes) it dropped below the initial level and remained at the same level during 3 days of observation.

Of visceral organs only the liver showed considerable radioactivity content which for Y^{90} did not vary significantly during the experiment. On the contrary, $CrP^{32}O_4$ pick-up by liver increased progressively during period of observation.

In other organs (spleen, kidney, lung), the organ/blood ratio of radioactivity was mostly within 1 to 2.8 range and only occasionally below 1; it was above 2 only for Y^{90} in the spleen during the first 30 minutes, and for chromic phosphate in all organs after 24 hours.

Accordingly, the pattern of radioisotopes distribution at various intervals after injection into the tail was outlined by radioactivity level in the tail and in the liver.

Discussion. The steady fall of radioactivity in the tail during the first hours after injection obviously resulted from removal of injected material by circulating blood. On the other hand, transient increase of radioactivity level 30 to 45 minutes after $CrP^{32}O_4$ injection and 1 to 3 hours after Y^{90} administration can be attributed to temporary obstacles of blood passage in tail vessels (blood clots, compression by swelling, etc.). This tentative explanation of the difference of the radioactivity in liver following administration of the two preparations would require dialysis as crucial evidence of the chromic phosphate preparation and a statement of the chemical dose of yttrium.

The abundant storage of Y^{90} in the injected tissue(1,2,4) is usually attributed to its fixation on tissue proteins. Considering conditions within the tail (highly vascularized narrow area with a minimum amount of loose connective tissue), an early release of yttrium-protein complex into the blood may be postulated for an explanation of the sharp drop in radioactivity after 30 minutes. The residual Y^{90} remained attached to tissue and underwent only a moderate loss afterwards.

The parallelism between decrease of $CrP^{32}O_4$ in the tail and its increase in liver suggested considerable translocation of material from site of injection into the liver. This process was excluded by the consistently stationary and low level of Y^{90} in the liver. It may be presumed that the Y^{90} released from the tail was taken by liver and spleen (increase of spleen radioactivity during its initial fall in the tail), but mostly by some tissue unrecorded in our experiment (bone(2)) or by excretion(1).

In these experiments, translocation of Y^{90} and colloidal $CrP^{32}O_4$ from site of injection was considerably more significant than in previously reported results(1,2,4) of intramuscular and subcutaneous administration. The pick-up of these isotopes by liver and on a small scale by other organs resembled the distribution pattern after their intravenous use. In fact, it could be roughly characterized as a delayed distribution pattern of intravenously injected $CrP^{32}O_4$ and Y^{90}. It should be remembered that in the ventral tail area the thin skin layer over the bone and cartilage frame encloses relatively large blood vessels with only a minimum of connective tissue between them. It is conceivable that in this area the injected material or its complex with proteins reached the blood stream more easily and more abundantly than from the dense muscle and the connective tissue provided only with small vessels.

Summary and conclusions. (1) Doses of 0.05 mc of $Y^{90}Cl^3$ solution and of $CrP^{32}O_4$ colloidal suspension in 0.05 or 0.1 ml were injected into ventral surface of tails in mice. At intervals of 1 minute to 3 days after injection, tails were serially amputated and preserved until 3rd day when every mouse was sacrificed; their organs (liver, lung, spleen, kidney), tails and heart blood (0.3 ml) were

emulsified, dried and tested for radioactivity content with a Geiger Tube Detector. (2) Results showed only a narrow range of radioactivity for each organ specimen calculated as a product of the average blood radioactivity in the same group of mice (amputated at the same time). (3) The calculated and tabulated data show that a significant proportion of $CrP^{32}O_4$ was translocated into the liver from the tail; for Y^{90} the initial storage in the tail was very high, but some translocation occurred, presumably by excretion. (4) The pattern of Y^{90} and $CrP^{32}O_4$ distribution in the body after injection into the tail was comparable to delayed results of intravenous injection; this resemblance was interpreted by high vascularization of the tail. (5) It was concluded that the method of serial tail amputation may be useful for outlining the dynamics of isotopes spread in bodies of experimental animals.

1. Kyker, G. C., *Am. J. Med. Sci.*, 1954, v227, 512.

2. Lahr, T. N., Olsen, R., Gleason, G. I., Tabern, D. L., *J. Lab. and Clin. Med.*, 1955, v4, 66.

3. Lewin, R., Hart, H. E., Greenberg, J., Spencer, H., Stern, K. G., Lazlo, D., *J. Nat. Cancer Inst.*, 1954, v15, 131.

4. Goldie, H., West, H. D., *Cancer Res.*, 1956, v16, 484.

5. Goldie, H., West, H. D., Jeffries, B. R., Butler, C. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 327.

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Effects of Ethyl Alcohol on Cardiac Output and Its Distribution in the Rat.* (24122)

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Ethyl alcohol is generally considered to increase peripheral blood flow. Particular interest has been directed to its possible effects as a coronary and cerebral vasodilator. The present studies were designed to determine whether alcohol influences the cardiac output and its distribution in the albino rat.

Methods employed in determination of cardiac output and regional uptake of indicators have been described(1,2). The conditions in which the fractional distribution of an indicator and the fractional distribution of the cardiac output correspond with each other have also been indicated(1,2). The cardiac output and the uptake fraction of all organs other than the brain were measured with Rb^{86}. Iodoantipyrine (I^{131}) was used in the brain studies. Female rats of Sprague-Dawley strain were used unless otherwise noted. Animals weighed 200-250 g after an 18 hour fast during which they were allowed free access to water. Each animal received 5 ml 20% alcohol by intraperitoneal injection. This amount was sufficient to produce light anesthesia. Five to 10 minutes after administration of the alcohol, either $Rb^{86}Cl$ or iodoantipyrine (I^{131}) was given in 0.4 ml by injection into an exposed femoral vein. Determination of cardiac output was made with Rb^{86} in animals in which the carotid artery had been catheterized with a short length of polyethylene tubing. Blood samples were collected at 0.67 second intervals, using a sample collector described elsewhere(3), and the arterial concentration curve of indicator constructed. Cardiac output was calculated as described(1). Other animals were employed for determination of Rb^{86} or iodoantipyrine (I^{131}) uptake by the organs at 15 or 30 seconds after administration. At these times, all organs other than brain have the same extraction ratio for Rb^{86} as the whole body; the fraction of Rb^{86} appearing in an organ, therefore, describes the fraction of cardiac output which perfuses it. Cerebral blood flow, which cannot be meas-

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TABLE I. Effect of Alcohol on Fractional Distribution of Iodoantipyrine (Brain) and Rb^{86} (Other Organs) at 15-30 Sec. after Injection in Rats.

	Pentobarbital			Alcohol		
	# rats	Avg	S.D.	# rats	Avg	S.D.
Heart	8	2.9	.5	20	3.8	.7 *
Lungs	12	3.7	.7	20	3.9	.9 *
Kidneys	8	18.7	5.5	20	12.7	3.0 *
Liver	12	7.6	1.0	16	7.4	1.6
Gut	12	19.3	4.3	16	17.4	3.6
Skin	12	6.4	.9	16	5.7	1.1
Spleen	12	1.3	.5	20	.7	.3
Brain†	14	.40	.10	16	.76	.17*

* Significant at 1% confidence level.
† Values converted to ml/g/min.

ured with Rb^{86}, was determined with iodoantipyrine (I^{131}) for which the brain and whole body have the same extraction ratio during the first 30 seconds after injection. Control animals were anesthetized with sodium pentobarbital 40 mg/kg unless otherwise noted.

Results. Cardiac output in alcohol treated rat. The average cardiac output in 5 rats receiving alcohol was 217 ml/kg/min (range: 180-243). The value did not differ appreciably from that found in 40 control animals of 200-210 g (225 ml/kg/min).

Rb^{86} uptake by organs of alcohol treated rats. The results in 32 alcohol treated rats are presented in Table I. Three organs, other than brain, show significant differences from values in normal controls. The coronary uptake fraction rose from 2.9 ± 0.5 (S.D.) in normals to 3.8 ± 0.7 in alcohol treated animals. Splenic uptake fell from 1.3 ± .5% to .7 ± .3 (S.D.) % of the injected dose in alcohol treated animals. The renal fraction fell after alcohol treatment. In normal animals the renal fraction was 18.7 ± 5.5 (S.D.) %; after alcohol, the value was 12.7 ± 3.0% of the injected dose.

Cerebral blood flow in alcohol treated rats and mice. Alcohol elevated calculated cerebral blood flow from 0.40 ± .10 ml/g/min to 0.76 ± .17 ml/g/min in rats. Flow values were calculated by multiplying body weight in kg by the average cardiac output/kg in each animal; this value was multiplied by fractional uptake of label by the brain in each animal and divided by brain weight in grams. Control values are based on rats anesthetized with sodium pentobarbital. Since the effect observed might have been due to a diminution from the normal, due to pentobarbital anesthesia rather than an increase from the normal due to alcohol, an attempt was made to administer the label by tail vein in unanesthetized animals. Because of the difficulty of making a successful venipuncture in the unanesthetized rat, a comparison of untreated, pentobarbitalized, and alcohol treated Albino Swiss mice was made. Anesthesia was induced with 1 mg of pentobarbital sodium given intraperitoneally or with 1 ml of 20% alcohol by the same route. Unanesthetized animals were loosely restrained in a 20 mm glass tube. Indicator injections of 0.2 ml were made into a tail vein. The animals were killed 10-15 seconds after injection of indicator.

The fractional uptake of iodoantipyrine by the brain in 4 conscious mice averaged 2.8% of the injected dose (range 2.1-3.6%). Three animals anesthetized with pentobarbital showed a cerebral uptake of label averaging 1.9% of injected dose (range 1.8-2.1%). Three animals which received alcohol had an average cerebral uptake of indicator of 4.2% of the injected dose (range 3.5-4.6%).

Discussion. The present experiments show that ethyl alcohol in the doses used is without effect on the cardiac output of the rat. Uptake of Rb^{86} by the heart is increased; renal and splenic uptake of this isotope is reduced. Alcohol increases cerebral uptake of iodoantipyrine. Since, in the experimental circumstances used, organ uptake parallels flow distribution, these findings suggest that alcohol increases blood flow to heart and brain while reducing blood flow to kidneys and spleen.

Increased coronary flow is in accord with clinical observation(4) that ethyl alcohol may alleviate anginal pain. Increase in coronary circulation has been more directly demonstrated after either intravenous or intracoronary injection of alcohol in the dog(5).

The reduction in the fractional uptake of Rb^{86} by the kidney was large. The failure of Smythe *et al.*(6) to detect renal blood flow changes in the dog may have been due to a species difference or to administration of pen-

tobarbital before the alcohol. The effect of alcohol on renal plasma flow in the absence of pentobarbital does not appear to have been investigated by bladder clearance methods.

The failure of alcohol to influence splanchnic uptake of Rb^{86} is in accordance with previous findings that alcohol does not influence splanchnic blood flow in the dog(6). The reported increases in splanchnic blood flow in man(7) may have been due to a species difference or to use of smaller doses in human subjects. The present studies show that in the rat distribution of splanchnic blood flow between hepatic artery and portal inflow other than the spleen is not altered by alcohol. The apparent effect of alcohol on the spleen may have resulted from alterations in the spleen due to pentobarbital(8) rather than from a true reduction in splenic blood flow due to alcohol.

The failure of alcohol to affect uptake of Rb^{86} by the skin appears at first to be inconsistent with the observations of increased skin blood flow by Abramson *et al.*(9) in man after alcohol. It should, however, be noted that these authors found that though the blood flow to the hand was increased by alcohol, the blood flow to arm and leg was unaffected, suggesting that the vasodilatation produced by alcohol may be restricted to a small portion of the skin.

The most striking changes produced by ethyl alcohol are on cerebral uptake of iodoantipyrine. Increased cerebral blood flow has been reported in stuporous subjects with blood alcohol levels of 320 mg %(10). Actual intake of alcohol by the subjects was unknown; neither was their nutritional status ascertained; smaller doses of alcohol given by vein in more normal subjects appear to be without effect on cerebral blood flow(10,11). From findings in the unanesthetized mice, it appears that alcohol increases cerebral blood flow above the normal value; although the value with pentobarbital is lower than that in the conscious mouse, the difference may be attributable to a response of the conscious animal to the procedure of handling, restraint, and injection rather than to any specific effect of the pentobarbital. In either case, alcohol increases cerebral flow fraction well above the conscious level or that observed in pentobarbital anesthesia.

Summary and conclusions. (1) Ethyl alcohol does not influence cardiac output of the rat in doses of 5 ml of 20% by volume solution in 200-250 g rats. (2) Blood flow values to the major organs calculated from the fractional uptake of Rb^{86} or iodoantipyrine (I^{131}) show the following: cutaneous blood flow (considering the skin as a whole) hepatic arterial inflow, portal flow other than splenic, and bronchial blood flow are unaffected by alcohol; coronary blood flow is increased 31%; renal blood flow is reduced 32%; cerebral blood flow is 90% greater in the alcohol treated than in the pentobarbitalized animal.

1. Sapirstein, L. A., *Am. J. Physiol.,* 1958, v193, 161.

2. Sapirstein, L. A., Hanusek, G. E., *ibid.,* 1958, v193, 272.

3. Rothe, C. F., Sapirstein, L. A., *Am. Heart J.,* 1954, v48, 141.

4. White, P. D., *Heart Disease,* 4th ed., Macmillan, 1951.

5. Lasker, N., Sherrod, T. R., Killam, K. F., *J. Pharm. Exp. Therap.,* 1955, v113, 414.

6. Smythe, C., Heineman, H. O, Bradley, S. E., *Am. J. Physiol.,* 1953, v172, 737.

7. Mendeloff, A. I., *J. Clin. Invest.,* 1954, v33, 1928.

8. Hahn, P. F., Bale, W. F., Bonner, J. F., Jr., *Am. J. Physiol.,* 1943, v138, 415.

9. Abramson, D. I., Zazeela, H., Schkloven, N., *Am. Heart J.,* 1941, v21, 756.

10. Battey, L. L., Heyman, A., Patterson, J. L., *J.A.M.A.,* 1953, v152, 6.

11. Hine, C. H., Shick, A. F., Margolis, L. L., Burbridge, T. N., Simon, A., *J. Pharm. Exp. Therap.,* 1952, v106, 253.

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Passive Immunization Against *Cryptococcus neoformans.** (24123)

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A number of investigators(1) have been successful in producing relatively potent antiserums in rabbits against *C. neoformans* by various immunization regimens, but none have employed these sera for *in vivo* studies. The present investigation was undertaken in an effort to extend our knowledge in this field.

Materials and methods. (1) *Antiserum Production.* Antisera were prepared against *C. neoformans,* isolates 3723, DU, LE, 1523 and RE by growing the organisms on asparagine medium(2) with 2% agar added. After 48 hours' incubation at 25°C, the cells were harvested, washed twice with saline (0.85% sodium chloride solution) suspended in 1% formalin and stored at 37°C for 12 hours. Finally, the cells were washed in saline, standardized by hemocytometer counts, and frozen at –27°C in 14 separate vials for each organism. Vaccination was performed by injecting 300 million cells on the first and second day intravenously (IV) into rabbits. Subsequent vaccinations were accomplished by injecting 250 million cells daily from the third through the fourteenth day. Each day one vial of each organism was thawed and used for that day's injection. The animals were bled 2 weeks following the last immunizing dose and the sera absorbed as follows:

(a) anti-RE absorbed with CN 1523 and 3723 cells
(b) anti-LE absorbed with CN 1523
(c) anti-1523 absorbed with CN RE
(d) anti-3723 absorbed with CN DU and LE cells
(e) anti-DU serum was not absorbed, since it had been determined that this serum was type specific(3).

Polyvalent antisera were prepared by mixing equal quantities of monovalent, absorbed sera. Agglutinin titers for each immunological component were reduced by approximately one-third. Finally, the mixed antisera were sterilized by filtration. (2) *Determination of agglutinin titer. Cryptococcus* cells were grown on asparagine medium for 48 hours and suspended in 0.5% formalin after 3 saline washes. The suspensions were standardized to McFarland No. 3 standard and mixed with serial dilutions of antiserum. Incubation was carried out in a water-bath for 2 hours at 37° C followed by storage at 5°C for 3 days. Titers thus obtained were as follows: anti-RE 1:320; anti-1523 1:320; anti-DU 1:320; anti-3723 1:160; and anti-LE 1:160. (3) *Virulence determination.* Virulence of the strains, CN DU and 3723, was titrated by intraperitoneal (IP) injections as previously reported(2) except that organisms were suspended in 5% hog gastric mucin. (4) *Antiserum administration.* Antisera were injected IP into white, male, Swiss (Rockland) mice in 0.5 ml doses daily for a total of 14 days following IP challenge with 10^6 cells of *C. neoformans* DU or $10^{3.8}$ of strain 3723. The antiserum injection on the first day followed the challenge dose by some 2 hours. The animals were observed until all had expired. (5) *Antiserum on phagocytosis* in vitro *and* in vivo. *In vitro* studies were initiated by isolation of mouse polymorphonuclear leucocytes (PMN)(4). Suspensions of organisms in silicone-coated vials were prepared to contain 10,000 cells of *C. neoformans* 3723 or DU and the following: (a) 0.4 ml PMN suspension and 0.4 ml normal rabbit serum (NRS). (b) 0.4 ml PMN suspension and 0.4 ml *C. neoformans* 3723 antiserum. Two 0.2 ml aliquots were transferred to separate vials, rotated at 55 rpm (37°C) for 40 minutes and plunged into ice water. Blood films were prepared from each vial, dried and stained by Wright's method. Recordings of degree of phagocytosis achieved were made by examination of smears to determine percent of

* Statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of Veterans Admin.

TABLE I. Effect of Passive Immunization on Mice Challenged with Selected Dose* of *C. neoformans* 3723.

Antibody†	No. of mice	No. of mice surviving at various intervals (Days) 5	10	20	30	40	50	70	90	110	130	150
RE-1523-LE	10	10	7	6	4	2	0					
DU-1523-LE	10	10	7	6	3	2	0					
DU-3723-LE	10	10	10	9	9	8	7	6	4	2	0	
DU-3723	10	10	10	9	9	9	7	7	5	2	0	
DU	10	10	8	6	2	1	0					
3723	10	10	10	10	10	9	8	8	6	3	2	0
Normal rabbit serum†	10	9	7	5	3	1	0					

* The selected dose was approximately one LD_{50} as determined in a prior experiment.
† Animals received daily inj. of antiserum or normal serum in control group for 14 days following challenge.

PMN containing ingested organisms. In view of the fact that *C. neoformans* RE could be separated into small and large capsule variants by plating(3); the above procedure was repeated employing both variants separately and mixed in 50/50 proportion. *In vivo* experiments were limited to studies in which pure preparations of small and large capsule variants of *C. neoformans* RE in gastric mucin were injected IP into separate groups of mice. Two hours later NRS or anti-RE serum was injected IP. After 24 hours, 2 mice from each group were injected IP with 0.5 ml saline, sacrificed 30 minutes later, and stained slides prepared from the peritoneal washing. The remainder of the animals received a dose of NRS or anti-RE serum. In this manner animals were sacrificed and additional inoculations were given for a period of 6 days. A number of mice were observed for 4 days after stopping passive immunization. Slide examinations of peritoneal washings and estimates of phagocytosis using the Wright procedure were made on all animals.

Results. Tables I and II quite clearly demonstrate that mice were protected against a lethal challenge dose of *C. neoformans* during the period of passive immunization by the action of antiserum. This protection is apparently type-specific. As soon as the antiserum is withdrawn, however, the animals develop the disease, although there appears to be a considerable delay in onset of the disease process.

To elucidate the mechanism of antiserum protection, ability of mouse PMN to phagocytize *Cryptococcus* cells was determined. *In vitro* experiments (Table III) show that a definite distinction must be made between small and large capsule variants of a given *Cryptococcus* culture. It is quite evident that the small capsule variants (SCV), whether

TABLE II. Effect of Passive Immunization on Mice Challenged with Selected Dose* of *C. neoformans* DU.

Antibody†	No. of mice	No. of mice surviving at various intervals (Days) 5	10	20	30	40	50	70	90	110	130	150	170	190
DU-1523-LE	10	10	10	10	9	8	8	6	5	4	2	2	0	
DU-3723-LE	10	10	10	10	10	9	8	7	6	5	3	2	0	
RE-3723-LE	10	10	10	10	9	8	7	6	5	4	2	2	0	
RE-1523-LE	10	10	10	9	8	7	6	5	4	2	2	1	0	
DU-3723	10	10	10	10	10	9	8	7	7	6	4	2	0	
RE-3723	10	10	10	10	10	9	8	8	7	6	5	3	0	
DU	10	10	10	10	10	9	9	8	8	6	5	3	1	0
3723	10	9	7	5	4	3	2	0						
Normal rabbit serum†	10	9	7	5	4	2	1	0						

* The selected dose was approximately one LD_{50} as determined in a prior experiment.
† Animals received daily inj. of antiserum or normal serum in control group for 14 days following challenge.

tested in a mixed culture, containing both large and small capsule varieties, or in pure culture (*C. neoformans* RE), are capable of being phagocytized in significant numbers. On the other hand, LCV are almost completely resistant to phagocytosis.

When phagocytosis of pure cultures of LCV and SCV of the RE strain were studied *in vivo* (Table IV), intraperitoneal phagocytosis of SCV decreased sharply in the presence of normal rabbit serum while phagocytosis of the LCV occurred rarely under any conditions. In the presence of homologous antiserum, however, there was no appreciable change in the phagocytic response to SCV during the immunization period, but a decrease in response was noted thereafter.

SCV and LCV strains of *C. neoformans* RE have been found to be stable variants on successive transfers *in vivo*. When SCV cultures were injected into animals, the size of their capsules increased. On subsequent transfer to solid or liquid media, SCV organisms were found exclusively. This observation has also been made(1) with other strains of cryptococci.

Discussion. Passive immunization has been demonstrated against a number of strains of *C. neoformans* by the use of mono and polyvalent, mixed antisera. The protection achieved in mice by homologous monovalent serum was always greater than that observed with divalent and trivalent sera. This difference is attributed to the lesser amount of homologous monovalent serum contained in the polyvalent types.

TABLE III. Ability of Mouse PMN's to Phagocytize *C. neoformans* Cells *In Vitro*.*

Organism	Capsule type ratio (small to large) Originals†	Final‡	Phagocytosis of Small capsule type	Large capsule type
			% §	
CN 3723	20/80	5/157	86.2	1
CN DU	5/80	3/163	90.0	1
CN RE	50/50	7/190	94.0	1
	100/0	12/0	92.8	
	0/100	0/382		1.3

* Results are avg of 2 exp.
† Indicates ratio in original culture.
‡ Indicates ratio of No. of uningested to ingested cells observed.
§ % calculated on basis of pure culture of variant.

TABLE IV. Ability of Mouse PMN's to Phagocytize *C. neoformans* RE, Small and Large Capsule Variants *In Vivo*, on Successive Days.*

Normal rabbit serum†		*C. neoformans* RE antiserum*	
Phagocytosis of		Phagocytosis of	
Small capsule type	Large capsule type	Small capsule type	Large capsule type
%			
56	3.0	60.5	3.5
49	3.5	59.5	4.0
38	4.5	60.0	6.0
21.5	6.5	62.5	7.0
9.5	5.5	65.0	6.5
4.5	6.5	66.5	6.5
2.5	6.0	66.0	5.5
2.5	6.0	58.5	6.0
2.0	6.5	39.0	6.0
2.0	7.0	26.5	5.0

* Results are avg of 2 exp.
† Serums were given daily for 2 days.

Evans(3) has classified the strains employed here by capsule type as follows: Type A—strains DU and RE; type B—strain 1523; type C—strain LE and untypable—strain 3723. On the basis of these immunization studies and the data of Evans it appears that the specificity of the protective response resides in the capsule.

It has been shown conclusively that isolated mouse PMN are capable of phagocytizing small, but rarely large capsule *Cryptococcus* variants (LCV). *In vivo* studies have confirmed this observation and, in addition, have demonstrated what appears to be a low grade opsonophagic response in one instance.

In spite of the apparent inability of mouse PMN to phagocytize a majority of LCV organisms, protection has been shown to cover the period of passive immunization. This observation suggests the possible presence of ablastins or other nonabsorbable humoral factors acquired as a result of immunization.

With these studies in mind it may be possible at the clinical level to employ type-specific antiserum to "contain" the disease, so that present-day antibiotics such as cycloheximide (Actidione†), even though toxic, can be employed in low doses or for short periods of administration.

† Upjohn Co., Kalamazoo, Mich.

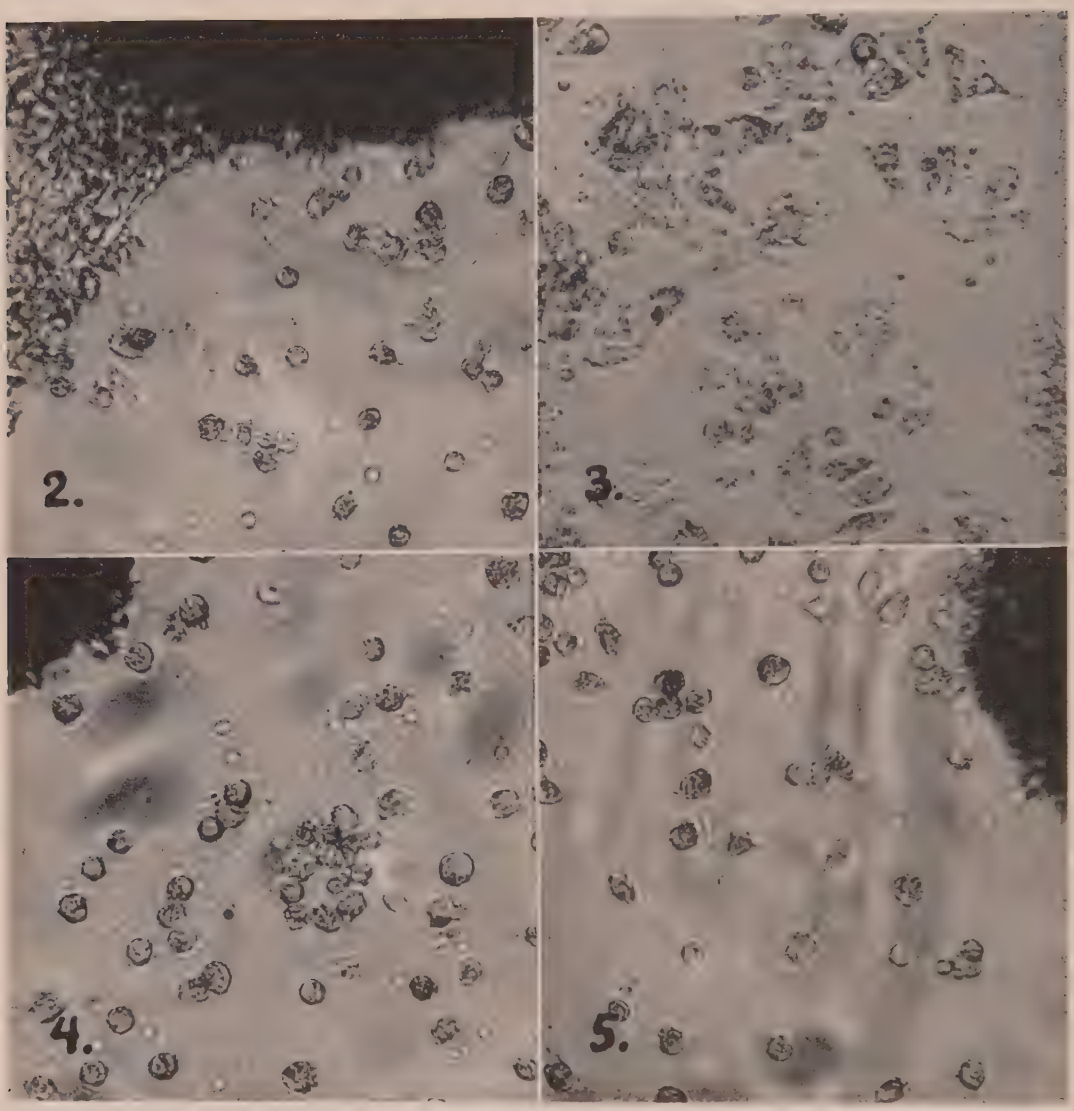

FIG. 2. Photomicrograph of spleen from skin-test positive guinea pig grown as normal control at 120 hr. ×100.

FIG. 3. Photomicrograph of spleen from skin-test positive guinea pig grown in presence of mumps virus antigen 72 hr after addition of antigen. ×100.

FIG. 4. Photomicrograph of spleen from skin-test positive guinea pig grown in presence of mumps virus antigen and cortisone acetate 72 hr after addition of antigen and 120 hr after addition of cortisone. ×100.

FIG. 5. Photomicrograph of spleen from skin-test positive guinea pig grown in presence of cortisone acetate 120 hr after addition of cortisone. ×100.

Note: All tissue cultures are 5 days old.

tion of 2 processes: (a) primary destruction of cells through direct cytolysis by actively multiplying virus; and (b) a secondary cytotoxic effect of mumps virus antigen on hypersensitive cells. Assuming this hypothesis to be true, then the data presented, within the limitation of the *in vitro* experimental situation, suggest a rationale for treatment of the complications of mumps virus infections in man with corticosteroids, on the basis that they block the hypersensitive component of the inflammatory reaction.

The effect of the corticosteroid compounds on the delayed hypersensitive state of tuberculosis has been well documented. *In vivo* studies(16-18) have demonstrated the ability of these compounds to alter the tuberculin reaction and Vollmer(20) noted reversal of tuberculin skin tests following treatment with steroids. Leahy and Morgan(21) reported suppression of the *in vitro* cellular reactivity to tuberculoprotein, indicating that the compounds have a direct action at the cellular level.

The observations presented here provide further evidence of the similarity of the delayed hypersensitive state in tuberculosis and mumps by demonstrating a protective action of corticosteroids on cytotoxicity of mumps viral antigen for hypersensitive cells *in vitro*.

Summary. Experimental mumps virus infections in guinea pigs are characterized *in vivo* by a positive delayed hypersensitive skin test reaction to viral antigen and *in vitro* by a cytotoxic effect of mumps virus antigen on cells from tissues cultivated from mumps-infected animals. Cortisone protects against this *in vitro* cytotoxicity of mumps virus.

1. Rich, A. R., Lewis, M. R., *Bull. Johns Hopkins Hosp.,* 1932, v50, 115.

2. Moen, J. K., Swift, H. F., *J. Exp. Med.,* 1936, v64, 339.

3. Moen, J. K., *ibid.,* 1936, v64, 943.

4. Gangarosa, E. J., Inglefield, J. T., Thomas, C. G. A., Morgan, H. R., *ibid.,* 1955, v102, 425.

5. Shea, J. D., Morgan, H. R., Proc. Soc. Exp. Biol. and Med., 1957, v94, 436.

6. Rich, A. R., *Pathogenesis of Tuberculosis,* Springfield, Charles C. Thomas, 1946.

7. Enders, J. F., Kane, L. W., Maris, E. P., Stokes, J., Jr., *J. Exp. Med.,* 1946, v84, 341.

8. Glasgow, L. A., Morgan, H. R., *ibid.,* 1957, v106, 45.

9. Petersdorf, R. G., Bennett, I. L., Jr., *Arch. Int. Med.,* 1957, v99, 222.

10. Zeluff, G. W., Fatherree, T. J., *Ann. Int. Med.,* 1957, v46, 852.

11. Stenberg, E. S., Jr., Berry, W. C., *U. S. Armed Forces Med. J.,* 1956, v7, 1414.

12. Bloodworth, A. F., Cohen, S. L., *ibid.,* 1956, v7, 285.

13. Solem, J. H., *Acta Med. Scandinav.,* 1954, v149, 341.

14. Risman, G. C., *J.A.M.A.,* 1956, v162, 875.

15. Smith, I. M., Bishir, J. W., *New England J. Med.,* 1958, v258, 120.

16. Long, J. B., Favour, C. B., *Bull. Johns Hopkins Hosp.,* 1950, v87, 186.

17. Harris, S., Harris, T. N., Proc. Soc. Exp. Biol. and Med., 1950, v74, 186.

18. Derbes, V. J., Dent, J. H., Weaver, N. K., Vaughan, D. D., *ibid.,* 1950, v75, 423.

19. Gell, P. G., Hinde, I. T., *Brit. J. Exp. Path.,* 1951, v32, 516.

20. Vollmer, H., *J. Ped.,* 1951, v39, 22.

21. Leahy, R. H., Morgan, H. R., *J. Exp. Med.,* 1952, v96, 549.

22. Hanks, J. H., Wallace, R. E., Proc. Soc. Exp. Biol. and Med., 1949, v71, 196.

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Increased Follicle-Stimulating Activity in the Plasma of Ovariectomized Rats.* (24125)

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Several investigators, using the technic of parabiosis, have been able to demonstrate that bilateral castration results in increased gonadotrophic activity in blood plasma of male and female rats(1). Emery(2) was able to show this increased activity by injection of castrate male serum into immature rats, and also reported on the basis of a few preliminary tests that serum from castrate, multiparous female rats showed gonadotrophic activity. Donor animals used were rats sacrificed over a 2 year period for various other purposes, and no attempt was made to standardize the post-operative period. The present study, in which immature hypophysectomized female rats

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have been used as test animals, reports the presence of follicle-stimulating activity in plasma as early as 7 days after castration and a gradual increase as the post-operative interval is extended to 4 months.

Methods. Virgin rats of the Long-Evans strain, averaging 233 g in body weight and 101 days of age, were bilaterally ovariectomized and maintained on stock diet XIV† for varying post-operative periods, *i.e.*, 1 and 2 weeks, 1, 2 and 4 months prior to autopsy. Normal animals of comparable age and body weight were used as onset controls. Blood was taken in heparinized syringe from abdominal aorta while animals were under light ether anesthesia, and was centrifuged under light mineral oil for 30 minutes at 3000 rpm. Minimal amounts of heparin had to be used as a slight excess resulted in subcutaneous hemorrhages and often death of test animal. The pooled plasma for each post-operative period was kept under oil at 4°C for 6 hours or less, so that each test animal was able to receive 4 equally divided doses at 2 hour intervals daily regardless of total amount of plasma injected. Fresh plasma was taken daily, although preliminary studies indicated that there was little if any loss of follicle-stimulating activity when plasma was kept at 4°C for several days. Castrate donors were examined macroscopically at time of autopsy to check completeness of ovariectomy. Plasma from castrated and normal rats was assayed for follicle-stimulating (FSH), and interstitial-cell stimulating (ICSH) activity in immature female rats hypophysectomized at 26 to 28 days of age and 12 to 20 days post-operative. Total doses of 4 cc, 8 cc, 16 cc, 24 cc, and 32 cc of donor plasma were given and the critical doses repeated at least once. Plasma was injected subcutaneously 4 times daily at 2 hour intervals as divided injections were necessary to insure survival when higher dosage levels were given. A 96 hour test period was used and autopsy performed 24 hours after last injection. Uteri and ovaries (minus oviducts were weighed and the ovaries fixed in Bouin's fluid for histological study. Completeness of hypophysectomy was checked by examination of the sella turcica under binocular microscope at time of autopsy. Histological responses for FSH activity and ICSH activity were determined by use of criteria described by Evans *et al.*(3). The minimal effective dose (MED) for follicle stimulation was that amount of plasma required to cause beginning antrum formation and increase in size of follicles above hypophysectomized control levels in two-thirds of test animals. These follicles are described as small-medium (sm) in the Tables. The MED for interstitial cell stimulation was that amount which caused partial repair of "deficient" interstitial tissue in two-thirds of the rats, *i.e.*, resumption of normal nuclear pattern in these cells with some increase in cytoplasm.

Results. The data presented in Table I show that FSH activity was detected in plasma of all rats after castration but not in plasma of normal onset controls at highest dosage level used. Within one week after removal of ovaries, injections of 32 cc (total dose) resulted in stimulation of follicular development in somewhat less than two-thirds of the test animals. Since the response was small follicles in one-half the test ovaries and small-medium in the remainder, this was considered to be slightly less than the minimal effective dose. A total dose of 32 cc of plasma from rats castrated for 2 weeks resulted in stimulation of follicular growth (small-medium follicles) in two-thirds of test animals, which by definition can be considered the minimal effective dose. When animals had been castrated for approximately one month, injection of 24 cc of plasma resulted in small follicles in some test ovaries and small-medium in others, a response less than the minimal effective dose, whereas a higher dosage (32 cc) showed a much greater response (small-medium to medium follicles) or more than the minimal effective dose. Therefore, the MED was considered to fall between 24 and 32 cc for this post-operative period. Similarly, with 2 months post-operative period, the MED was considered to fall between 16 and 24 cc, and a

† Stock diet XIV is composed of 68.5% ground whole wheat, 10% fish meal, 10% alfalfa leaf meal, 5% casein, 5% fish oil (Sardiline), and 1.5% iodized NaCl. Lettuce given *ad libitum* twice weekly.

TABLE I. Follicle Stimulating Activity in Blood Plasma of Normal and Castrated Female Rats.

Post-operative period, days	Total plasma dose, cc	No. test animals*	Uterus wt, mg†	Ovaries‡		FSH activity	FSH activity, MED/100 cc plasma
				Wt, mg	Follicles		
0	0	14	17	7	s	Neg.	
	8	9	16	7	s	"	
	16	9	18	7	s	"	
	24	6	17	9	s	"	
	32	7	18	10	s	"	
7	0	12	17	8	s	Neg.	<3
	24	8	16	8	s	"	
	32	12	19	9	s-sm	<MED	
14–17	0	9	16	7	s	Neg.	3
	24	6	19	7	s	"	
	32	6	17	8	sm	MED	
31–38	0	13	15	7	s	Neg.	3–4
	24	11	19	8	s-sm	<MED	
	32	11	25	11	sm, few m	>MED	
60–65	0	10	14	7	s	Neg.	4–6
	8	6	16	6	s	"	
	16	8	16	7	s	"	
	24	8	37	14	m, few ml	>MED	
	32	6	48	14	m-ml	"	
117–128	0	9	16	8	s	Neg.	6–12
	4	8	17	8	s	"	
	8	7	19	8	s	"	
	16	8	18	11	sm, few m	>MED	
	24	7	40	15	m, few ml	"	
	32	6	46	14	sm-m, few ml	"	

* Immature female rats, hypophysectomized 26-28 days, 12-20 days post-operative.

† Uterus was dissected free of oviducts and drained of fluid before weighing.

‡ s = small, m = medium, l = large—refer to size of follicles measured with calibrated eyepiece micrometer. Standards are those used by Wooten *et al.*(4). s = up to 375 μ, sm = 450-500 μ, m = 550-650 μ, ml = 700-750 μ, l = 800-1000 μ.

MED—minimal effective dose.

4 month post-operative period showed a further increase as the MED dropped to a point somewhere between 8 and 16 cc of plasma. The gradual increase in FSH activity with increased post-operative period can easily be seen when approximate FSH activity per 100 cc of plasma is calculated (Table I).

As judged by morphology of interstitial tissue in hypophysectomized recipients, no evidence for ICSH activity was found in plasma of either intact or castrate animals. However, increased uterine weights indicating production of estrin (which many investigators consider requires the presence of both ICSH and FSH), were observed when plasma from castrate animals 1 to 4 months post-operative was given.

The results confirm the findings of Emery (2) and the brief mention by Goto(5) and Martins(6) that increased gonadotrophic activity in blood of castrate rats can be demonstrated by direct injection of plasma into test animals. The findings are also in agreement with those obtained through the technic of parabiosis, *e.g.*, Biddulph *et al.*(7), and show that increased gonadotrophic activity in castrate female rats is predominantly follicle-stimulating. The latter investigators found evidence for increased FSH activity in the circulation as early as 3 days post-operative. In addition, our data demonstrate a gradual but progressive increase in circulating FSH activity with increasing post-operative periods up to 4 months.

It should be mentioned that growth promoting activity, as measured by the standard tibia test,‡ was present when 24 and 32 cc levels of both normal and castrate rat plasma

‡ Right tibias of hypophysectomized test animals

were tested in immature hypophysectomized recipients. The growth-stimulating potency of normal rat plasma was equivalent to that recently reported by Contopoulos *et al.*(9).

Summary. Follicle-stimulating activity was detected in blood plasma of adult female castrate rats as early as 7 days post-operative by injection of plasma into immature hypophysectomized female rats for a 4-day period. Follicle-stimulating activity continued to increase slowly but progressively as the post-operative interval increased to a 4 months' period. With this bioassay method no direct evidence for circulating interstitial cell-stimulating activity was found at the highest dosage level tested.

were split mid-sagitally, fixed in formol, and stained with $AgNO_3$(8). The uncalcified portion of proximal epiphyseal cartilage was measured with micrometer eyepiece and average widths of 200 μ or greater (i.e., approximately 50% increase over uninjected control widths) were considered evidence for growth-stimulating activity.

1. Finerty, J. C., *Physiol. Revs.,* 1952, v32, 277.
2. Emery, F. E., *Am. J. Physiol.,* 1932, v101, 246.
3. Evans, H. M., Simpson, M. E., Tolksdorf, S., Jensen, H., *Endocrinology,* 1939, v25, 529.
4. Wooten, E., Nelson, M. M., Simpson, M. E., Evans, H. M., *ibid.,* 1955, v56, 59.
5. Goto, N., *Arch. f. exp. Path. u. Pharm.,* 1922, v94, 124.
6. Martins, T., *Inst. Oswaldo Cruz, Memorias Suppl.,* 1929, v10, 242.
7. Biddulph, C., Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 92.
8. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. M., *Endocrinology,* 1949, v45, 455.
9. Contopoulos, A. N., Simpson, M. E., *ibid.,* 1957, v61, 765.

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Relationship of Fluoride and Dietary Fats to Serum Cholesterol in Rats.* (24126)

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It was shown(1) that doses of 0.5, 1 or 2 mg fluoride as aqueous solutions of sodium fluoride daily did not alter normal serum cholesterol level in the rat. Since Miller and Phillips(2) have found an enhancement of fluoride toxicity in rats on a diet containing 15% cottonseed oil, further work pertaining both to fluoride toxicity and serum cholesterol in which different sources of dietary fat are used seemed indicated.

Methods. A total of 150 weanling male Sprague-Dawley strain rats were divided equally into 5 series. Series A was divided into 3 groups, all receiving a stock sucrose diet. Group I animals received no fluoride in their drinking water, Group II received fluoride as sodium fluoride at concentration of 30 μg F/ml in the drinking water, and Group III received 2 mg of fluoride daily (as sodium fluoride) by stomach tube. Each of the 4 remaining series was divided similarly into 3 groups and received the same amount of fluoride as Series A animals either in drinking water or by stomach tube, but the type of dietary fat was changed. The animals in Series B received cottonseed oil, those in Series C corn oil, Series D Crisco, and Series E lard. Fat was added at a level of 20%. Animals in Group I and III of each series received a fluoride low (F = 0.05 μg/ml) drinking water *ad libitum*. Composition of the experimental diets which were also available *ad libitum* are summarized in Table I. All animals were housed individually in raised screen cages in air-conditioned room and were weighed twice each week throughout experiment of 10 weeks. Serum cholesterol was determined at beginning of experiment and at weekly intervals (except for 6 and 9 week period) by methods previously described(1).

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† Post-doctorate fellow on leave from Mainz Univ., Germany.

TABLE I. Composition of Experimental Diets (g %).

Component of diet	Animal series A	B	C	D	E
Sucrose	60	45	45	45	45
Vit. test casein	30	30	30	30	30
Cottonseed oil	5	20			
Corn "			20		
Crisco				20	
Lard					20
Salt mixture*	4	4	4	4	4
Vit. " *	1	1	1	1	1
Oleum percomorphum†					

* For composition of salt and vitamin mixture, see *J. Nutr.*, 1954, v54, 481.
† 15 drops/kilo diet.

Results. Body weight gains for animals receiving various experimental diets (Table II) indicate that all diets produced relatively good weight gains.

Serum cholesterol values (Table III) indicate that there is a steady increase in serum cholesterol with age of animals. Type of fat or amount of fluoride has little effect on the serum cholesterol level.

In Table IV the weekly mean serum cholesterol data are presented. Again the steady increase in serum cholesterol with the age of animals is evident.

Table V shows mean serum cholesterol level obtained by averaging the 10 weekly analysis in each dietary fat series, in the control group, and in the fluoride groups I and III. Vertical averages in this Table indicate the effect of varying fluoride levels on serum cholesterol; horizontal averages show the effect of fat on serum cholesterol level. The similarity of values in any direction demonstrates that neither fluoride nor any of the diet variables caused significant changes in serum cholesterol level.

TABLE II. Weekly Weight Gain of Animals Receiving Various Experimental Diets. (In each series 30 animals equally divided into 3 groups.)

Dietary group	Wt (g) Initial*	5 wk	10 wk
A Sucrose	92	208	244
+ 30 μg F/ml	98	194	252
+ 2 mg F/da	90	196	238
B Cottonseed oil	96	195	252
+ 30 μg F/ml	96	191	250
+ 2 mg F/da	103	215	272
C Corn oil	118	240	320
+ 30 μg F/ml	107	217	261
+ 2 mg F/da	110	232	281
D Crisco	120	228	293
+ 30 μg F/ml	112	203	254
+ 2 mg F/da	104	204	226
E Lard	117	212	231
+ 30 μg F/ml	126	227	260
+ 2 mg F/da	114	200	230

* Data were obtained at weekly intervals and will be provided upon request.

TABLE III. Effect of Various Experimental Diets on Serum Cholesterol Level in the Rat. (In each series 30 animals equally divided into 3 groups.)

Series	Weekly serum cholesterol levels (mg %) 1 wk*	5 wk	10 wk
A Sucrose	98 ± 4†	117 ± 3	124 ± 7
+ 30 μg F/ml	114 ± 2	121 ± 3	114 ± 5
+ 2 mg F/da	97 ± 3	110 ± 5	108 ± 6
B Cottonseed oil	100 ± 2	111 ± 4	116 ± 5
+ 30 μg F/ml	98 ± 4	113 ± 4	150 ± 5
+ 2 mg F/da	92 ± 5	100 ± 4	137 ± 4
C Corn oil	100 ± 4	121 ± 8	138 ± 2
+ 30 μg F/ml	94 ± 4	104 ± 6	140 ± 7
+ 2 mg F/da	94 ± 4	103 ± 5	130 ± 5
D Crisco	84 ± 1	119 ± 2	129 ± 7
+ 30 μg F/ml	95 ± 4	125 ± 4	150 ±11
+ 2 mg F/da	78 ± 5	134 ± 6	135 ± 5
E Lard	110 ± 5	109 ± 5	124 ± 8
+ 30 μg F/ml	81 ± 4	108 ± 4	115 ± 2
+ 2 mg F/da	98 ± 6	92 ± 3	110 ± 5

* Determinations were made at weekly intervals and will be provided upon request.
† Stand. dev.

TABLE IV. Increase in Serum Cholesterol as a Function of Age (mg %, 30 Rats/Series).

Series	Weeks 1	2	3	4	5	7	8	10
A Sucrose	103	109	113	112	116	105	118	115
B Cotton-seed oil	97	105	108	105	108	108	166	134
C Corn oil	96	98	102	109	109	138	114	136
D Crisco	86	90	105	111	126	123	108	138
E Lard	96	115	124	98	103	155	138	116
Avg	96	103	110	107	112	126	129	128

Discussion. As judged by body weight increases, the effect of 20% fat in the diet does not seem to increase toxicity of fluoride when compared to a similar diet containing only 5% dietary fat. Furthermore, in all cases the animals which received fluoride weighed as much if not more than their respective controls not receiving fluorides, except in Series C and D where corn oil and Crisco, respectively, were the sources of

TABLE V. Effect of Fat and Fluoride Level on Serum Cholesterol (mg %, 30 Rats in Each Series, Equally Divided into 3 Groups).

	Series A	B	C	D	E	
Group	Sucrose	Cottonseed oil	Corn oil	Crisco	Lard	Avg
I No fluoride	110	113	115	112	123	115
II 30 μg F/ml	116	120	115	112	115	115
III 2 mg F/da	109	116	108	109	117	112
Avg	112	116	113	111	118	

dietary fat. Even in animals receiving the latter 2 diets the final weight difference of the groups receiving fluoride as compared to their controls does not suggest an increased fluoride toxicity since, unfortunately, the initial weight of the fluoride animals was less than the respective controls, thus making actual final weight comparison less meaningful. For animals receiving 2 mg of fluoride a day, this is approximately 600 times as much fluoride as would be received by a 170 lb man receiving 1 mg fluoride a day, an amount considered optimal for dental health.

It is evident that at times during experimental period there are what appear to be pronounced increases in cholesterol level, but upon subsequent measurement the high values return to approximately the mean for the nonfluoride groups. Fluorides, whether administered in drinking water or by stomach tube are without significant effect.

These data suggest that the animals receiving diets containing 20% fat have a tendency to higher serum cholesterol levels than animals receiving 5% fat-containing control diet. However, in animals receiving 20% cottonseed oil, the increase occurred only during the eighth week, and by the end of the tenth week the serum cholesterol in the control 20% cottonseed oil group was less than the control sucrose group (containing 5% cottonseed oil). Similarly, the increase in serum cholesterol in the corn oil and Crisco group occurred during the tenth week. The final serum cholesterol levels for the lard groups were quite similar to the sucrose groups. Thus, these data serve to illustrate the large, but presumably normal, variation which occurs between groups during an experiment. When a statistical analysis was carried out, no significant differences were found between any of the groups in this study.

Obviously, one is concerned about the role of cholesterol metabolism and its suggested relationship to cardio-vascular disease(3). Since fluoridation is an accepted anticaries public health procedure one must be doubly concerned not to increase cholesterol level by use of fluorides. These data, as well as those obtained in a previous study(1) would suggest that the use of fluorides, even at very high levels as in this study, is not associated with changes in cholesterol metabolism. Some caution is needed in relating these data to those obtained in the human since the rat is considered by some workers to be moderately resistant to slight changes in serum cholesterol level.

Summary. Serum cholesterol level was determined in 5 series of rats receiving either different fats in their diets or different fats plus fluoride to determine whether the use of fluoride is associated with an increased serum cholesterol level. After administration of 2 mg of fluoride daily throughout a 10-week period there was no significant change in serum cholesterol level of rats regardless of their dietary fat source or the presence of fluoride. Comparison of body weight gains of the various groups fails to suggest that any of the fats used resulted in increased fluoride toxicity.

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1. Buttner, W., Muhler, J. C., *J. Nutrition,* 1957, v63, 263.

2. Miller, R. F., Phillips, P. H., *ibid.,* 1955, v56, 447.

3. Dawber, T. R., Moore, F. E., Mann, G. F., *Am. J. Pub. Health,* 1957, v47, 4.

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Strontium/Calcium Ratios in Parts of Rat Femur.* (24127)

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Behavior of strontium in the animal body has become of particular interest in relation to possible hazards from atomic fission debris. The body burden of radiostrontium in terms of body calcium, attained after ingestion of contaminated material over long periods of time, will be governed by Sr/Ca ratio of the intake and by the over-all discrimination between strontium and calcium. Of the many metabolic processes in which such discrimination occurs, this investigation deals only with the discrimination observed in particular parts of a long bone, as studied in rats using double tracer experiments with Ca^{45} and Sr^{89}.

Methods. Young female rats of Wistar strain, weighing 160-200 g, and grown on normal stock diet, were given a single intraperitoneal injection of a mixture of Ca^{45} and Sr^{89}. The 0.5 ml dose contained about 8 microcuries each of Ca^{45} (carrier-free) and Sr^{89} (containing less than 10% Sr^{90}), and 2.8 mg of Ca as $CaCl_2$. Animals were killed at intervals of 15 minutes, 30 minutes, 1 hour, 2, 4, 8, 24, and 48 hours after injection, with 3 to 6 rats in each group. A terminal caval blood sample was taken with most rats. Both femurs were removed, cleaned of adherent muscle, cut transversely into thirds, pooled to make comparable samples of proximal ends, distal ends, and shafts for each group of animals. After weighing, each bone sample was dried at 105°C for 24 hours and then ashed at 600°C for 24 hours in a muffle furnace. The weighed ash was ground and aliquots taken to prepare oxalated samples with a plastic-tube-and-cup assembly as described by Comar(1). The 2 isotopes were measured by usual methods for radioassay of mixtures based on differential absorption of the beta emission. An aliquot of a certain dilution of the injection solution was oxalated with 40 mg of normal bone ash and carriers exactly like the experimental samples and constituted the standard against which all measurements were compared. All samples were counted after a 21-day delay to allow the unknown amount of Sr^{90} present to reach equilibrium. Blood serum calcium was determined by standard volumetric permanganate titration methods adapted for our purposes, the sample re-oxalated and a radioassay done. Radioactivity in serum was recorded as counts/minute of Ca^{45} and Sr^{89}/mg of serum calcium. The results are expressed as strontium/calcium Observed Ratio (OR bone/injection solution) as suggested by Comar *et al.*(2): OR bone-injection sol. = $\frac{Sr^{89}/Ca^{45} \text{ in bone}}{Sr^{89}/Ca^{45} \text{ in inj. soln.}}$.

Results. The Observed Ratios of the 3 parts of femur, *viz.*, distal ends, proximal ends, and shafts, are shown in Table I and graphically illustrated in Fig. 1. A maximal strontium content appeared early in all samples with the shaft samples, indicating actual discrimination against calcium. During the following 48 hours, the OR's gradually fell in all bone samples but remained consistently higher in samples of the shaft. Specific activity of both Ca^{45} and Sr^{89} (counts/minute of Ca^{45} or Sr^{89}/mg stable Ca) reached a maximum in distal and proximal growth areas between 4 and 8 hours after injection, and was always higher than in samples of shaft bone.

In the right portion of Fig. 1, sharpest rise in OR was in femur shaft and serum, with drop for serum a rapid one from 15 minute to 4 hour interval, while the shaft OR showed a second peak at 4 hours before it gradually fell off. The initial peak in the shaft OR curve at 30 minutes is questionable since the S.D. for that group of animals was large; actually the curve could have been a gradual rise from 15 minutes to 4 hours, with the fall beginning at the 4 hour interval.

The Observed Ratio appeared to fit a straight line with time from 4 to 48 hours. Accordingly, straight lines were fitted and

* This work was supported by Grant-in-Aid of U. S. Atomic Energy Com.

TABLE I. Sr^{89}/Ca^{45} Observed Ratios in Pooled Femur Parts, and in Blood Serum at Intervals after a Single Intraperitoneal Injection of Ca^{45} and Sr^{89}.

Time	Distal ends		Shafts		Proximal ends		Serum	
15 min.	*.820	.116	.864	.112	.820	.115	.732	
30	.901	.031	1.157	.328	.896	.081	1.000	.954
1 hr	.794	.064	.940	.063	.788	.115	.938	.036
2	.735	.097	.907	.074	.734	.138	.887	.100
4	.651	.014	1.026	.019	.627	.050	.460	.114
8	.588	.069	.975	.034	.627	.034	.406	.054
24	.576	.003	.835	.148	.565	.044	.444	.063
48	.487	.088	.67	.070	.516	.040	.334	.018

* Mean and stand. dev. of mean.

the following slopes (with upper and lower 95% confidence limits for the true slope) were recorded (Table II).

A straight line was not a good fit to Sr^{89}/Ca^{45} for blood serum, the data indicating slight evidence for a decrease in Sr^{89}/Ca^{45} with time; but the decrease was not significant because of the variability. Slopes and confidence limits for bone show that there was a significant negative decrease of Sr^{89}/Ca^{45} with time for distal femurs, femur shafts, and proximal femurs. The slope in all cases represented the average decrease in Sr^{89}/Ca^{45} for each hour from 4 to 48 hours. The decrease for femur shafts was significantly more (at 95% level) than that for proximal femurs and was very nearly significantly more than that for distal femurs and whole femurs.

Discussion. Accumulated experimental evidence (Bauer *et al.*(3); Comar, *et al.*(4)) indicates that with regard to skeletal accretion and exchange, there is no marked difference between calcium and strontium. The differences in retention which appear in both single and multiple dose studies were due to preferential excretion of strontium by the kidneys (Talmadge(5), MacDonald(6)). The observed ratios reported in the present single dose experiments show an expected discrim-

TABLE II.

	Lower 95% confidence limit	Slope	Upper 95% confidence limit
Distal femur	–.0045	–.0032	–.0019
Femur shafts	–.0120	–.0081	–.0043
Proximal femurs	–.0040	–.0027	–.0014

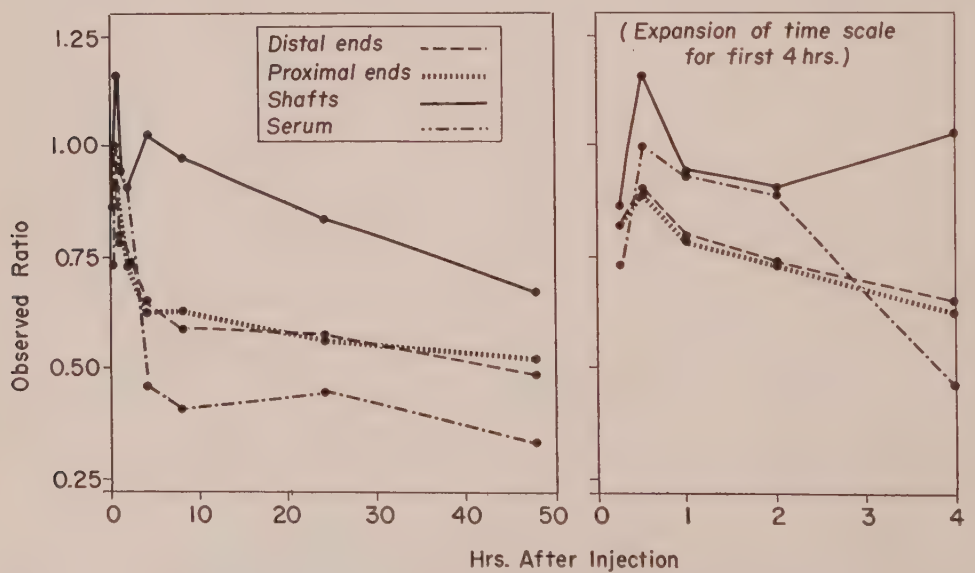

FIG. 1. Sr^{89}/Ca^{45} observed ratios for parts of femur and for blood serum plotted against time after a single inj. of a mixture of Sr^{89} and Ca^{45}.

ination against strontium with time. It is inferred that the observed discrimination occurs in the kidney and that endogenous excretion *via* the gut is negligible in this case. The point to which attention is directed is the differential observed between parts of long bones in the rat under the experimental conditions used. The more rapid turnover of the radioisotopes in processes of accretion, exchange, and resorption in the epiphyseal ends of the femur as contrasted with the less actively growing shaft leads to a preferential loss of strontium in the ends of the femur as compared with the shaft. It is conceivable that vascular arrangements in the epiphyses as compared with the shaft play a role in the discrimination observed.

Summary. After intraperitoneal injection of a mixture of Ca^{45} and Sr^{89} in the rat, the Sr/Ca Observed Ratio in parts of a long bone, the femur, was obtained 15 minutes to 48 hours after injection. A maximal Sr content appeared in bone within 30 minutes, with the shaft showing discrimination against Ca; during the next 48 hours the Observed Ratios fell but remained consistently higher in shaft portions of the bone. Data on blood Sr/Ca ratios are also presented.

1. Comar, C. L., *Radioisotopes in Biology and Medicine,* McGraw-Hill, 1955.

2. Comar, C. L., Wasserman, R. H., Nold, M. M., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 859.

3. Bauer, G. C. H., Carlsson, H., Lindquist, B., *Acta Physiol. Scand.,* 1955, v35, 66.

4. Comar, C. L., Wasserman, R. H., Progress in Mc. Energy, ser. VI, v1, 1956, *Biolog. Sc.,* Pergamon Press, London.

5. Talmadge, R. V., Schooley, J. C., Comar, C. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 413.

6. MacDonald, N. S., Noyes, P., Lorick, P. C., *Am. J. Physiol.,* 1957, v188, 131.

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Effect of Phenethylbiguanide on Adrenal Function and Responsiveness as Measured by ACTH Test.* (24128)

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The quest for an oral hypoglycemic agent has received considerable impetus since reintroduction of the aryl-sulfonylureas by Franke and Fuchs(1), extending the work that Janbon(2) and Loubatieres(3) had begun 13 years earlier. Unger, Freedman and Shapiro (4), reviewing a group of potentially hypoglycemic drugs, discovered N B′ Phenethylformadinylimourea (DBI)†, a biguanide. DBI has been used by Pomeranze(5,6), Williams, *et al.*(7), and in our own clinic (unpublished) in treatment of hyperglycemia in man. It lowers blood sugar concentration in patients with diabetes mellitus but the moderately severe side effects of nausea and anorexia have precluded its continued use in many. The mechanism by which biguanides act is unknown. Since adrenal steroids can affect blood glucose level by increasing gluconeogenesis and decreasing glucose utilization, it was believed that one action of DBI might involve the blockade of adrenal steroid production. Our studies were designed to measure the effect of DBI on adrenocortical secretion of hydrocortisone and demonstrate either a decrease in or a blockade of adrenal cortical activity by comparing baseline plasma hydrocortisone levels with levels after DBI, and by comparing baseline responses of adrenal gland to pituitary stimulation (ACTH test) to responses to ACTH following DBI therapy.

Methods. The 6 subjects, all on wards of St. Luke's Hospital had recognized diabetes mellitus for 1 month to 20 years and were well controlled on 20 to 80 units of insulin/day. These patients received DBI for 8 to

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† Dr. L. Freedman, U. S. Vitamin Corp., generously supplied the DBI.

TABLE I. Adrenal Responsiveness to ACTH in 6 Patients with Diabetes Mellitus before and after Treatment with DBI.

Patient (yr & sex)	Duration of diabetes mellitus	Insulin dosage (units)	Duration DBI treatment (days)	Daily dosage DBI (total mg)	Pre-DBI ACTH test			Post-DBI ACTH test		
					Baseline*	2 hr	4 hr	Baseline	2 hr	4 hr
					γ %					
R.C. 21 ♂	1 mo	40 (NPH)	15	250 (no insulin)	13	33	41.1	24	30	37
P.M. 17 ♂	9 yr	60 (lente)	12	150 (40 u lente)	13	40.1	49.3	6	29	41
I.W. 85 ♀	17 "	35 (PZI)	15	100 (no insulin)	30	49	50	18.5	32	101
A.B. 21 ♂	10 mo	40 (NPH)	8	250 (no insulin)	24.3	27	44	42	55	61
I.Q. 36 ♀	3 yr	80 (NPH)	8	275 (30 u regular)	15	29	42	23	26.3	26.3
E.S. 67 ♀	12 "	DBI initial therapy	15	150 (no insulin)	25		59	7.3		28.6

* Plasma 17,21-dihydroxy-20-ketosteroid.

15 days. Two patients required insulin in reduced amounts for control of hyperglycemia during treatment with DBI, while 4 were maintained on drug alone. All patients had an ACTH test as outlined by Christy, Wallace and Jailer(8) before and after treatment with biguanide. The test consists of baseline, 2 hour and 4 hour plasma hydrocortisone levels measured during infusion of 25 units of aqueous ACTH in 500 cc of isotonic saline. 17, 21-dihydroxy-20-ketosteroid concentrations in plasma were determined by the method of Silber and Porter(9). By this method 80% of the chromogenic reacting material is hydrocortisone. DBI added to pooled plasma in concentrations varying 0.05 mg to 0.3 mg % did not alter values for hydrocortisone as determined by Silber-Porter technic.

Results. In pre-treatment ACTH tests, the average baseline plasma hydrocortisone level was 20 γ/100 ml (range 13-30 γ/100 ml), and after ACTH administration the average level was 47.6 γ/100 ml (range 41.1-59 γ/100 ml). After DBI therapy the average baseline value was 20.1 γ/100 ml) (range 6-42 γ/100 ml) and after ACTH administration the average level was 49.1 γ/100 ml (range 26.3-101 γ/100 ml). (Table I). Patients (P.M., I.W., E.S.) showed a depression of post-treatment baseline hydrocortisone levels while 3 (R.C., A.B., I.Q.) showed elevated baseline levels. Patients (R.C., I.Q.) had a diminished response to ACTH following biguanide therapy, 2 (P.M., A.B.) showed essentially no change, I.W. had an increased response while patient E.S. had an increased response but depressed baseline level. All these values are within normal limits for this test with the exception of I.W. who showed a high 4 hour level.

Discussion. Gluconeogenesis and glucose release by liver are of major importance in maintaining normoglycemia(10). Although Tyberghein and Williams(11,12) demonstrated marked reduction in liver glycogen content in the guinea pig following DBI administration, further work(12) indicates another effect on hyperglycemia aside from that of hepatic carbohydrate metabolism as shown by the success of Nielsen, *et al.*(13) in inducing hypoglycemia with DBI in hepatectomized guinea pigs maintained with glucose.

A decrease in adrenal steroidogenesis and concomitant decrease in gluconeogenesis might explain why Williams, *et al.*(12), found a decrease in both liver glycogen and urinary nitrogen in animals fed alanine and DBI. While increased peripheral utilization by insulin enhancement or some other mechanism could account for Nielsen's findings. Our results in human subjects failed to show any consistent pattern of alteration in plasma hydrocortisone baseline concentration or ability

of adrenal to respond to ACTH. This would seem to eliminate suppression of adrenal cortical activity as a mechanism whereby DBI lowers blood glucose.

Summary. Adrenal function was studied in 6 diabetic patients with hyperglycemia controlled by DBI (N B′ phenethylformadinylimourea) or by DBI and insulin. DBI administration did not significantly affect baseline plasma hydrocortisone levels or response to ACTH. These results appear to eliminate adrenal steroid suppression as a possible mode of action of this biguanide.

1. Franke, H., Fuchs, J., *Deut. Med. Wochschr.*, 1955, v80, 1449.

2. Janbon, M., Chaptal, J., Uedel, A., Schaap, J., *Montpellier Med.*, 1942, v21-22, 441.

3. Loubatieres, A., *Compt. rend. soc., biol.*, 1944, v138, 766.

4. Unger, G., Freedman, L., Shapiro, S. L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 190.

5. Pomeranze, J., Fujiy, H., Mooratoff, G. T., *ibid.*, 1957, v95, 193.

6. Pomeranze, J., *J. Clin. Endo. and Metab.*, 1957, v17, 1011.

7. Williams, R. H., Tanner, D. C., Odell, W. D., *Diabetes*, 1958, v7, 87.

8. Christy, N. P., Wallace, E. Z., Jailer, J. W., *J. Clin. Invest.*, 1955, v34, 899.

9. Silber, R. H., Porter, C. C., *J. Biol. Chem.*, 1954, v210, 923.

10. Long, C. N. H., *Ciba Foundation Colloquia on Endocrinology*, Little Brown Co., Boston, 1953, vVI, 136.

11. Tyberghein, J. M., Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 29.

12. Williams, R. H., Tyberghein, J. M., Hyde, P. M., Nielsen, R. L., *Metabolism*, 1957, v6, 311.

13. Nielsen, R. L., Swanson, H. E., Tanner, D. C., Williams, R. H., O'Connell, M., *A.M.A. Arch. Int. Med.*, 1958, v101, 211.

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Metabolism of dl-Adrenaline-2-C^{14} in the Human*† (24129)

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Little information is available on metabolic products of adrenaline in the human. After intravenous infusion of adrenaline only 1 to 3% of the amount infused can be recovered in the urine as adrenaline or a conjugate of adrenaline(1,2,3). Following intravenous infusion of adrenaline-2-C^{14}, Resnick and Elmadjian(4) recovered 90% of radioactivity in urine within 30 hours; when adrenaline-methyl-C^{14} was infused, about 34% of the radioactivity was recovered. Schayer(5) previously found similar results in the rat and was able to distinguish 6 radioactive peaks by paper chromatography. Armstrong and McMillan(6), and Armstrong, McMillan and Shaw (7) identified 3-methoxy-4-hydroxymandelic acid (MOMA) as a product of noradrenaline metabolism in humans. In addition, Axelrod (8), and Axelrod *et al.*(9) found 3 methyl-O-adrenaline (metadrenaline) and 3 methyl-O-noradrenaline (normetadrenaline) and their corresponding glucuronides in urine of the rat.

This report describes a procedure which utilizes a combination of filter paper and ion exchange chromatography, to quantitatively separate the bulk of radioactive compounds which appear in urine after intravenous infusion of dl-adrenaline-2-C^{14}.

Materials. The sources of materials were as follows: dl-adrenaline-2-C^{14}, specific activity 1.09 mc/m mole from Tracerlab, Boston, Mass.; Amberlite IRC-50, XE-64 from Rohm and Haas Co., Philadelphia, Pa.; Dowex 1-2X, 200-400 mesh, from Dow Chemical Co., Midland, Mich. *Methods.* Infusion of dl-Adrenaline-2-C^{14} and Collection of Urine

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‡ Established Investigator of Am. Heart Assn.

Specimens: Healthy males aged 20 to 35 years were given an intravenous infusion of 5 μcuries (4.5 μmoles) of the labeled adrenaline in 200 ml of physiological saline over a one-hour period. Urine was collected at end of infusion period, at one hour intervals for the next 5 hours, and at 6 hour intervals for subsequent 18 hours. An aliquot of each specimen was pooled to give a 24-hour urine sample. Radioactivity in each specimen was determined with a thin window Geiger tube (16 c.p.m. background) and corrected to infinite thinness by reference to a previously prepared absorption curve. *Separation of urinary metabolic products.* A modification of the method previously reported by Kirshner, Klingman, and Goodall(10) was used. An aliquot of urine (5 to 10 ml) containing 15,000 to 30,000 counts/minute is passed through a 3 X 1 cm column of Amberlite IRC-50(11). The column is then washed with 15 ml of water. The effluent and wash are combined and saved. The IRC-50 column is then eluted with 20 ml of 0.5 N acetic acid. The eluate is evaporated to dryness and the residue dissolved in 0.5 ml of water. This solution is then chromatographed for 15-18 hours on Whatman No. 1 filter paper using n-butanol saturated with N HCl as the solvent. After drying, the paper is cut into 1 cm strips; each strip is eluted with water and an aliquot plated out for assay of radioactivity. Three radioactive peaks are found. One of the peaks corresponds to adrenaline; another corresponds to metadrenaline and the third peak has not yet been identified. The adrenaline and metadrenaline peaks account for 80 to 90% of the radioactivity found on the paper. The combined effluent and wash from the IRC-50 column is then passed through a 10 X 1 cm column of Dowex-1 acetate. The column is washed with 15 ml of water and the effluent and wash are combined and assayed for radioactivity. About 3% of total activity of the 24 hour urine specimen is obtained in this effluent and wash. The Dowex-1 column is then attached to an automatic fraction collector and eluted with 0.3 M ammonium acetate at pH 4.8. Three ml fractions are collected. The course of the fractionation is followed by assaying an aliquot of each third sample for radioactivity. When radioactivity in the eluate returns to background, usually after 80 to 90 ml of the buffer have passed through the columns, the column is eluted with 1 M ammonium acetate at pH 4.8. The fractionation is again followed by assaying each third fraction for radioactivity and again a sharp radioactive peak is obtained. When the radioactivity in the eluates again returns to background, the column is eluted with 3.0 M ammonium acetate pH 4.8. The fractionation is again followed similarly and a third peak is obtained. *Identification of Dowex-1 Eluates. 0.3 M Eluate.* This fraction is largely an acid labile conjugate of metadrenaline. After heating for 15 minutes with 1 N hydrochloric acid at 100°, 60% of the radioactivity originally present can be recovered as metadrenaline. The metadrenaline was isolated from the hydrolysate by absorption on IRC-50 and identified by paper chromatography using butanol-1 N HCl and isopropanol-ammonia-water as solvents. The conjugate is not a glucuronide since incubation of either whole urine or the Dowex eluate with either bacterial (Sigma) or animal (Warner-Chilcott) β-glucuronidase for 24 hours caused no increase in the metadrenaline fraction. However, the conjugate was hydrolyzed by incubation with Mylase P (Nutritional Biochemicals). In a parallel experiment in which rats were given an intraperitoneal injection of adrenaline-2-C^{14}, a radioactive fraction was obtained from the 24 hour urine specimen which was hydrolyzable by β-glucuronidase. This radioactive fraction was identified as metadrenaline glucuronide by isolation of radioactive metadrenaline after treating the fraction with β-glucuronidase. The metadrenaline glucuronide obtained from rat urine can be differentiated from the acid labile conjugate of metadrenaline obtained from human urine by ion exchange and filter paper chromatography. *1 M eluate.* Radioactive fractions were combined and concentrated. An aliquot was chromatographed on Whatman No. 1 paper in n-butanol saturated with N HCl and in isopropanol-ammonia-water (8:1:1). Indicator amounts of 3-methoxy-4-hydroxy mandelic acid were added in each instance. After de-

TABLE I. Distribution of Radioactivity in 24 Hr Urine following Infusion of Dl-adrenaline-2-C^{14}. Figures represent % of total radioactivity which appeared in urine. The .3 M, 1.0 M and 3.0 M eluates contain respectively, conjugated metadrenaline, 3-methoxy-4-hydroxymandelic acid and 3,4-dihydroxymandelic acid.

	% recovery of infused dose	IRC-50 fractions				Dowex-1 fractions				
		Total	Adr.	Metadr.	Unknown	.3 M eluate	1.0 M eluate	3.0 M eluate	Dowex-1 effluent	% recovery of radioactivity in urine
	70	11	4	5	2	43	27	16	2	99
	65	12	4	5	1	52	23	9	3	99
	75	14	5	6	2	39	29	8	3	93
	72	10	4	5	1	46	24	8	4	92
	78	10	3	5	1	35	31	14	3	93
	76	12	4	4	1	35	29	18	2	96
Avg	73 ± 5	12 ± 1	4 ± 0	5 ± 0	1 ± 0	42 ± 7	27 ± 3	12 ± 4	3 ± 0	95 ± 3

veloping for 15 to 18 hours, the paper was dried and sprayed with diazotized p-nitroaniline followed by 10% Na_2CO_3. After drying, the paper was cut into 1 cm strips, eluted with water, and an aliquot of each eluate assayed for radioactivity. In each case radioactivity was found only in the area coincident with the added 3-methoxy-4-hydroxymandelic acid. *3 M eluate.* The radioactive fractions were treated similarly to those of the 1.0 M eluate. A sharp radioactive peak corresponding to the position of 3,4-dihydroxymandelic acid (DOMA) was obtained in the butanol-N HCl solvent; in the isopropanol-ammonia-water solvent, 2 radioactive peaks were obtained. However, destruction of the DOMA which had been added as a marker was observed.

Results. Table I presents the data from experiments in which aliquots of 24 hour urine specimens obtained from 6 different subjects were assayed. The average recovery of $73 \pm 5\%$ of the infused dose is lower than that obtained by Resnick and Elmadjian(4) in humans and by Schayer(5) in rats. Of the total radioactivity in urine, 4% is due to adrenaline. This amount corresponds to 3% of the infused dose which is in accord with previous reports(1,2,3). Radioactive adrenaline could be detected only in the urine samples collecting during the infusion period and during the first and second hours after the infusion.

No previous data on excretion of metadrenaline or its conjugate in human urine are available. The results reported here for excretion of metadrenaline in human urine are approximately the same as those for the rat (9). In addition, Axelrod *et al.*(9) reported that, in the rat, 20% of an intraperitoneal injection of adrenaline is excreted in the urine as the glucuronide of metadrenaline. We have been able to confirm this finding in the rat. In the human, the acid labile conjugate of metadrenaline accounts for about 30% of an infused dose of adrenaline. This acid labile conjugate does not appear to be a glucuronide since it is not hydrolyzed by β-glucuronidase and differs chromatographically from the metadrenaline glucuronide obtained from the rat.

Recently Armstrong and McMillan(6) identified 3-methoxy-4-hydroxymandelic acid as a metabolic product of noradrenaline and estimated that 30% of an infusion of noradrenaline was converted to this acid. In the 6 experiments reported here, 27% of the radioactivity which appeared in the urine after an infusion of dl-adrenaline-2-C^{14} was recovered as 3-methoxy-4-hydroxymandelic acid.

Dihydroxymandelic acid has been previously found in human urine(12), however, no estimates of the amounts of this acid derived from adrenaline metabolism were made. The figures presented for this compound are tentative pending more rigorous identification of the material eluted from the Dowex-1 column.

The radioactive material of human urine which appears in the Dowex-1 effluent has not yet been characterized. It is interesting to note that in rat urine, the glucuronide conjugate of metadrenaline is found in the Dowex-1 effluent.

Summary. A procedure is described which utilizes a combination of filter paper and ion exchange chromatography to separate the urinary metabolic products of adrenaline. After intravenous infusion of dl-adrenaline-2-C^{14} in 6 human subjects, 73 ± 5% of infused radioactivity was recovered in 24 hour urine. Radioactivity which appeared in urine was distributed among various metabolic products as follows: adrenaline, 4%, 3-methyl-O-adrenaline, 5%, 3-methyl-O-adrenaline conjugate, 42 ± 7%; 3-methoxy-4-hydroxymandelic acid, 27 ± 3%; 3, 4-dihydroxymandelic acid, 12 ± 4%; 2 unidentified fractions, 4%.

ADDENDUM: Recent work on the separation procedure has resolved the 0.3M eluate and the 3.0M eluates each into two fractions. Preliminary work indicates that the metadrenaline conjugate accounts for about 80% of the radioactivity in the 0.3M eluate and one unidentified compound accounts for the remainder. In the 3.0M eluate, 3,4-dihydroxymandelic acid accounts for 30 to 50% of the radioactivity. This work is still in progress and will be reported at a future date.

1. von Euler, U. S., Luft, R., Sundin, T., *Acta Physiol. Scand.,* 1954, v30, 249.

2. Elmadjian, F., Lamson, E. T., Neri, R., *J. Clin. Endocrinol. and Metab.,* 1956, v16, 222.

3. Resnick, O., Elmadjian, F., *Fed. Proc.,* 1957, v16, 462.

4. ———, *J. Clin. Endocrinol. and Metab.,* 1958, v18, 28.

5. Schayer, R. W., *J. Biol. Chem.,* 1951, v189, 301.

6. Armstrong, M. D., McMillan, A., *Fed. Proc.,* 1957, v16, 146.

7. Armstrong, M. D., McMillan, A., Shaw, K. N. F., *Biochim. et Biophys. Acta,* 1958, v25, 422.

8. Axelrod, J., *Science,* 1957, v126, 400.

9. Axelrod, J., Inscoe, J. K., Senoh, S., Witkop, B., *Biochim. et Biophys. Acta,* 1958, v27, 210.

10. Kirshner, N., Klingman, G. I., Goodall, M. C., *Fed. Proc.,* 1958, v17, 87.

11. Kirshner, N., and Goodall, McC., *J. Biol. Chem.,* 1957, v226, 207.

12. von Euler, U. S., Recent Progress in Hormone Research, 1958, vXIV. Academic Press, Inc., New York.

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Plasma Cholesterol in Growing Chicken as Influenced by Dietary Protein and Fat.*† (24130)

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In the human, Ahrens *et al.*(1), Beveridge *et al.*(2), and others have indicated a relationship between degree of saturation of ingested fat and resulting serum cholesterol level. Studies with "normal" experimental animals (those not receiving dietary cholesterol or other cholesterolemic agents) have shown essentially no differential response to dietary fat(3). Hegsted and coworkers(4) presented evidence of a significant negative correlation between the product of the essential fatty acids and total saturated fatty acids (EFA x TSFA) of dietary fats and serum cholesterol level. Serum cholesterol level can be experimentally elevated not only through dietary cholesterol but also by feeding low protein diets(5,6,7). Our objective was to evaluate the effect of level and type of dietary fat on plasma cholesterol in growing chickens receiving either 1) low protein diet plus 0.3% cholesterol or 2) high protein diet with 2% cholesterol. Since differences in serum cholesterol as a result of varying the dietary fat (as observed by Hegsted *et al.*) might be related to differences in absorption of dietary cholesterol it was of particular interest to find out whether the low protein induced hypercholesterolemia would respond differen-

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TABLE I. Effect of Type and Level of Fat on Plasma Cholesterol, Weight Gain, and Feed Consumption of Growing Chicks Fed 2 Levels of Protein and Cholesterol.

	8% protein + .3% cholesterol					25% protein + 2% cholesterol				
		Fat					Fat			
Measurements	Level, %	Olive (55)*	Corn (491)	Coconut + cottonseed§ (1290)	Coconut + safflower§ (2120)	Level, %	Olive (55)	Corn (491)	Coconut + cottonseed§ (1290)	Coconut + safflower§ (2120)
Plasma cholesterol, mg %	3	738 ± 76†	744 ± 85	739 ± 64	504 ± 43	3	220 ± 19	177 ± 16	273 ± 92	153 ± 6
	6	779 ± 42	600 ± 74	782 ± 66	846 ± 60	6	347 ± 30	158 ± 10	224 ± 22	196 ± 12
	9	829 ± 56	816 ± 42	679 ± 65	866 ± 82	9	212 ± 31	386 ± 44	254 ± 38	251 ± 18
Avg wt gain, g/16 days	3	40 ± 11 †	51 ± 6	24 ± 8	51 ± 12	3	217 ± 11	222 ± 14	213 ± 10	179 ± 6
	6	12 ± 6	63 ± 8	40 ± 9	19 ± 6	6	202 ± 8	183 ± 6	201 ± 10	175 ± 12
	9	24 ± 7	35 ± 8	39 ± 12	42 ± 7	9	187 ± 14	186 ± 10	213 ± 16	225 ± 10
Avg feed consumption, g/16 days	3	278‡	273	244	284	3	423	426	440	395
	6	207	312	280	247	6	389	378	389	388
	9	240	256	267	264	9	397	365	406	426

* Product of essential fatty acids × total saturated fatty acids. † Mean ± stand. error. ‡ Avg consumption determined from total feed consumption of each lot. § Equal amounts of both fats were used.

tially to various fats.

Methods. White Leghorn cockerels were reared for 2 weeks on a practical starting ration, at which time they were divided into groups of 8 chicks/treatment. Two-week-old chicks were used to avoid any effects from unabsorbed yolk material. Percentage composition of basal diet was as follows: isolated soybean protein (Drackett Assay Protein C-1) 10, DL-methionine 0.10, mineral mix (8) 5.34, fiber 3, choline Cl 0.20, B vit. mix (8) 0.15, Vit. A, D and E mix(8) 0.10, glucose 72.61, cholic acid 0.20, cholesterol 0.3. The basal diet served as the low protein diet while the high protein diet was formulated by addition of 20% isolated soybean protein, 0.2% DL-methionine and 1.7% cholesterol to the basal diet at the expense of glucose. By including 3 levels of 4 different fats or fat-combinations, at the expense of glucose, into each of these 2 diets, 24 different treatments were obtained. Fats and fat combinations were chosen to cover a wide range of the product EFA x TSFA as given by Hegsted *et al.*(4). The design of the experiment is found in Table I. The birds were maintained on experimental diets for 16 days in electrically heated wire cages. They were weighed individually at beginning and end of experimental period. Group feed consumption was recorded. Blood samples were taken at end of experimental period by heart puncture using a heparinized syringe. After centrifugation to separate plasma and cells, 0.05 ml aliquots of plasma were taken for cholesterol analysis by modification of the method of Zlatkis(9) using stable iron reagent of Rosenthal *et al.*(10).

Results. At each of the 2 protein levels there were essentially no effects from either the type or level of dietary fat (Table I). It is thus apparent that Hegsted's finding in the rat is not applicable to the chicken. The hypercholesterolemia induced by combination of low protein level and small amount of dietary cholesterol was not affected by any of the fats. The observed plasma cholesterol levels on high protein diet were essentially normal, indicating ability of the chicken to adjust to large amounts of dietary cholesterol in the presence of otherwise adequate diet. By con-

trast Katz and Stamler(11) induced marked cholesterolemia in the growing chick by addition of the same level of cholesterol (2%) and 5% cottonseed oil to a diet containing 18% protein. This level of protein, in the authors' experience, is inadequate for proper metabolism of the absorbed cholesterol.

Weight gains and feed consumption in our study (Table I) indicate uniformity at each protein level. This is of particular interest since the caloric density of the diets varies considerably between the 3 and 9% levels of dietary fats. The uniformity of feed consumption might well be a factor in uniformity of the plasma cholesterol values.

Summary. The effects on plasma cholesterol level of 4 fats each supplied at 3 levels in a high and a low protein diet were studied in the cholesterol-fed chicken. On the low protein diet a marked hypercholesterolemia was observed which was not affected by type of fat studied or level of supplementation. On the high protein diet, plasma cholesterol levels were essentially normal regardless of source and level of dietary fat. Since the fats used represented a wide range in the product EFA x TSFA, a lack of relationship between this product and plasma cholesterol level in the growing chicken is indicated.

1. Ahrens, E. H., Insull, W., Blomstrand, R., Hirsch, J., Tsaltas, T. T., Peterson, M. L., *The Lancet,* 1957, v1, 943.

2. Beveridge, J. M. R., Connell, W. F., Mayer, G. A., DeWolfe, M. S., *J. Nutrition,* 1955, v56, 311.

3. Aftergood, L., Deuel, H. R., Jr., Alfin-Slater, R. B., *ibid.,* 1957, v62, 129.

4. Hegsted, D. M., Gotsis, A., Stare, F. J., *ibid.,* 1957, v63, 377.

5. Kokatnur, M., Rand, N. T., Kummerow, F. A., Scott, H. M., *Circulation,* 1956, v14, 962.

6. Nikkila, E. A., Ollila, O., *Acta Pathol. Microb. Scand.,* 1957, v40, 177.

7. Moyer, A. W., Kritchevsky, D., Logan, J. B., Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 736.

8. Fisher, H., Johnson, D., Jr., *J. Nutrition,* 1956, v60, 261.

9. Weiss, H. S., Fisher, H., *ibid.,* 1957, v61, 267.

10. Rosenthal, H. L., Fluke, M. L., Buscaglia, S., *J. Lab. and Clin. Med.,* 1957, v50, 318.

11. Katz, L. N., Stamler, J., *Experimental Atherosclerosis,* Charles C. Thomas, Springfield, Ill., 1953.

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Use of Ionophoretic Mobility Equilibria in Fractionation of Mixtures Containing Indoles.* (24131)

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Chromatographic separations of indoles in urine samples are often complicated by interfering substances which mark the spot, distort the Rf value and spot size, or cause streaking (1,2). It is also quite difficult to separate certain alkaloids from pigments and other "interfering substances" in urine by the conventional methods of paper electrophoresis; however, good separations are practicable by the mobility equilibrium method (unpublished data, 1952). The phenomenon of mobility equilibrium (M.E.) was first described in open-hanging-strip electrophoresis by Durrum (3). Separations by this method are time-consuming and require high current flow with volatile electrolyte systems. Some of the theoretical considerations presented in this paper have also been discussed by Macheboeuf, *et al.*(9). The purpose of this report is to describe a less time-consuming method in which low current flow may be utilized; the use of horizontal or vertical types of open paper electrophoresis units is practicable; and volatile or non-volatile electrolytes may be employed. It is thus possible to separate neutral molecules and charged ions (amphoteric and non-

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amphoteric) on a single strip of paper.

Methods. Two procedures have been used to determine M.E. values for compounds under specific conditions. *Strip test.* Strips of Whatman 3 MM filter paper (1½" x any convenient length) are moistened with the electrolyte. The samples are applied as indicated in Fig. 1A. The electric current is then applied for 1 to 2 hours in an open type paper electrophoresis unit. The spots are detected by their fluorescence in ultraviolet light, subsequent to drying the paper in an oven containing an atmosphere of formaldehyde. This treatment, however, precludes good color development with p-dimethyl-amino-benzaldehyde and with 1-mitroso-2-naphthol. Alternatively, the spots are detected by immersing or spraying the dried paper with the latter reagents to produce colored spots on a faint yellow background(4,5). The spots are then encircled, their total movement measured (Figs. 1B & 1C), and plotted as in Fig. 1D. The intercept of the locus of movement and the locus of origin delineates the M.E. Its numerical value is obtained by measuring the distance from this point to the center line. A negative or positive sign is then affixed. *Sheet test.* Samples are applied to a sheet of Whatman 3 MM filter paper as depicted in Fig. 2, and processed as were the strips. The terminal points of migration are connected at their centers. The intercept of this line and the locus of origin represents the M.E.

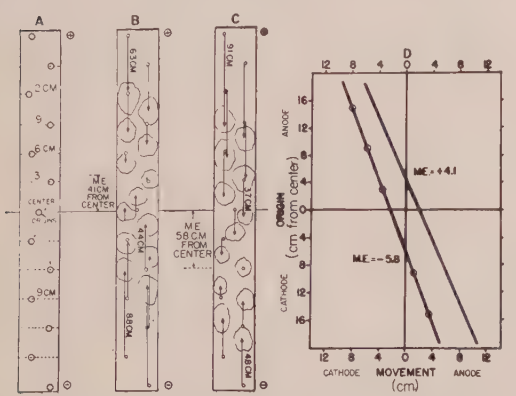

FIG. 1. Determination of M. E. values by the Strip Test, for 5-hydroxyindoleacetic acid (−4.1) and 5-hydroxytryptamine (+5.8), with a vertical type electrophoresis unit (apex height, 24 cm), in 0.05 M phosphate buffer, pH 7.0.

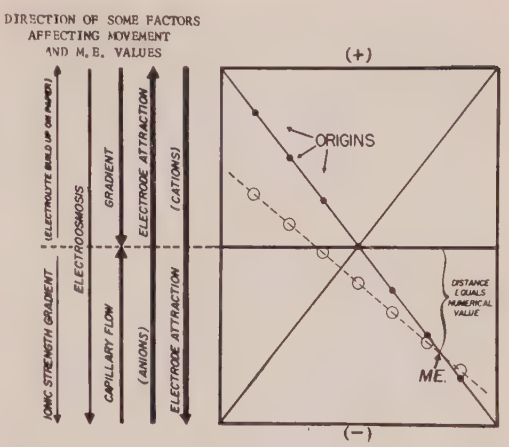

FIG. 2. Schematic representation of direction and relative magnitude of some factors on movement and M. E. of a cation in the Sheet Test. Compare with Fig. 3.

Results. The strip test is convenient and more economical for a single compound, while the sheet test is most practical for resolving mixtures. Fig. 3 illustrates an application of the method in fractionation of a mixture of urea and 3 indoles (5-hydroxy-tryptamine, 5-hydroxy-indoleacetic acid and 5-hydroxytryptophan). In this experiment the sheet test reveals: (1) the M.E. position of a substance in the mixture (#3, zone I and #9 zone III), (2) the position on the paper at which 2 or more substances may be separated from one another in the shortest interval of time, without resolving the other components in the mixtures (1A from 1B zone I 5A from 5D zone II), and (3) the zone in which best separations, with minimal spot spreading occurs (#4, zone II). Use of the sheet-test provides the maximal amount of information on the paper electrophoretic behavior of the components in a mixture in a minimal amount of time, and makes the use of the usual trial and error methods unnecessary. The determined M.E. value or a specific zone on the paper may then be selected for routine fractionations in accordance with experimental needs. Fig. 2 depicts the direction and relative magnitude of the major factors affecting movement of these compounds and producing the mobility equilibrium phenomenon. The ions are attracted by electrodes of opposite charge. Their mobilities are inversely proportional to

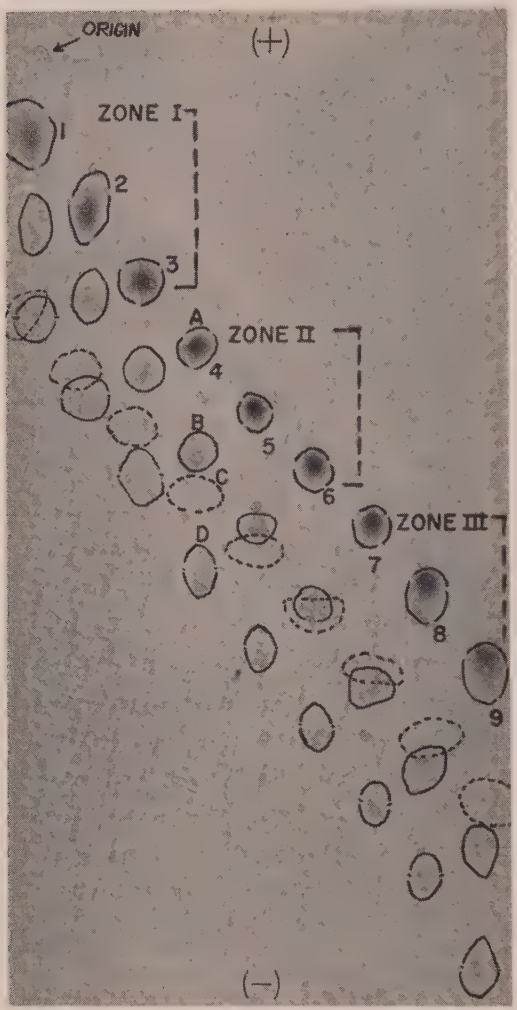

FIG. 3. Results of the Sheet Test performed on mixture of 5-hydroxyindoleacetic acid (A), 5-hydroxytryptophan (B), urea (C), and 5-hydroxytryptamine (D), using 0.05 M barbital buffer, pH 8.6, and a horizontal electrophoresis unit.

the ionic strength of the electrolyte, approaching the Y-axis asymptotically(6). In these studies a discreet stepwise decrease in numerical M.E. values was demonstrated by progressively increasing the starting concentration of the electrolyte. Evaporation as a sequel of the heat generated by the electric current passing through the filter paper, causes capillary forces to constantly draw fluid from the electrolyte compartments, with a flow rate gradient being set up toward the drier center. As evaporation of the solvent ensues, especially for non-volatile electrolyte systems, the amount of solute on the paper increases in such a manner that highest concentration is found midway between the electrodes. Filter paper or cellulose is structurally an aggregate of submicroscopic colloidal particles in the form of a reticulum. A certain number of carboxyl groups are usually found in filter paper, which may ionize, depending upon the pH. When alkaline electrolytes are used the ionized groups give the network of cellulose a negative charge; however, since the components of the reticulum are fixed and cannot migrate toward the anode in the imposed electrical field, the fluid in the interstices of the network moves toward the cathode. This movement of fluid accounts for the cathodal displacement of non-charged components in a mixture (*e.g.* urea, #5C zone II, is more cathodally displaced in this experiment than 5-hydroxytryptophan, #5B zone II, which possesses a weak negative charge at pH 8.6). Electroosmosis is the major factor responsible for the difference in absolute values of mobility equilibria for cations and anions of

TABLE I. Some Factors* Affecting the Movement and Mobility Equilibria of Indoles in Open-Type Paper Electrophoresis.

Factor	Movement			
	Cations		Anions	
	Anodal	Cathodal	Anodal	Cathodal
1. Electrode attraction	+	+	+	+
2. Capillary flow gradient	+	—	—	+
3. Electroosmosis	+	+	—	—
4. Ionic strength gradient (due to electrolyte build-up on paper)	—	+	+	—
5. pH	Significant effect on amphoteric compounds			
6. Adsorption and diffusion	Minimal			

* The factors are listed in order of decreasing magnitude.
+ = enhancement, — = inhibition.

otherwise molecularly similar species. In moderate concentrations of buffers, the compounds used in these experiments migrated as round spots (#3 zone I), indicating minimal adsorption(7). Spot spreading usually was most salient near the electrolyte compartments (compare #1A and #9A with #5A, Fig. 3). Lateral diffusion at M.E. occurs in experiments of long duration. Changes in the pH of the buffer in the electrolyte vessels are minimal even when volatile electrolytes are used(3). Positive ions exhibit maximal and negative ions show minimal mobilities on the anodal side of center. Conversely, cation mobility is lowest, and anion mobility highest on the cathodal side. Advantage may be taken of these regions of increased and decreased mobility by using longer strips of paper in a standard open-type electrophoresis unit. The extra length may be folded into the electrolyte vessels or counterpoised rollers may be inserted into these compartments. If the unresolved components of a mixture migrate into a region where differences in mobility are very slight (*e.g.* #5B & C zone II or #1C & D zone I) the paper may then be moved toward that electrode where mobility differences are greater (*i.e.* anode for #5 and cathode for #1) and electrophoresis resumed. This principle has been used to separate certain alkaloids from urinary pigments (unpublished data 1952) and more recently by Wolfson(8) and by Lederer *et al.*(8) to separate normal and abnormal adult hemoglobins. These investigators made successive analyses on the cathodal side of center. The zone of mobility equilibrium for a given ion: (1) represents the resultant of several forces, (2) occurs on the side of least movement (cathodal for cations, anodal for anions), and (3) is independent of the point of application of the sample. Its numerical value is equivalent to the distance from the center of this zone to the center line of the paper, with appropriate sign affixed.

Summary. (1) A method for rapidly determining the mobility equilibrium of a compound is described. The procedure provides an analytical rather than the conventional trial and error method for determining paper electrophoretic characteristics of the components in a mixture. (2) Some factors affecting ion movement and mobility equilibria in open-type paper electrophoresis units are discussed. (3) An application of the method in separating urea and several indole derivatives is presented.

1. Twarog, B. M., Page, I. H., *Am. J. Physiol.*, 1953, v175, 157.

2. Page, I. H., *Physiol. Rev.*, 1954, v34, 563.

3. Durrum, E. L., *J. Colloid Sci.*, 1951, v6, 274.

4. Rapport, M. M., *J. Biol. Chem.*, 1949, v180, 961.

5. Udenfriend, S., Titus, E., Weissbach, H., *ibid.*, 1955.

6. McDonald, H. J., Urbin, M. C., Williamson, M. B., *J. Colloid Sci.*, 1951, v6, 236.

7. Kunkel, H., Tiselius, A., *J. Gen. Physiol.*, 1951, v35, 89.

8. Lederer, M., *Introduction to Paper Electrophoresis,* Elsevier, New York, 1955, p43.

9. Macheboeuf, M., *Bull. Soc. Chim. Biol.*, 1953, v35, 346.

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Blood Plasma Proteins in Fetal Goats and Sheep.* (24132)

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The electrophoretic patterns of fetal plasma proteins have been reported to differ from their maternal counterparts mainly by the presence of a special protein, fetuin(1), and in some species by the lack of γ-globulins

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(2,3). The present investigation deals with distribution of fetal plasma proteins in 2 closely related species, the goat and sheep, and with the quantitative changes which individual plasma proteins undergo during fetal development.

Methods. Fetuses of goats and sheep of various ages were delivered by cesarean section and samples of heparinized blood taken from the umbilical vessels. After centrifugation, the plasma was removed and the total protein content determined refractometrically. Paper electrophoresis was carried out in the hanging strip type (Durrum) cells, using barbiturate buffer with a pH of 8.6 and ionic strength of 0.085. With 8 paper strips per cell, a constant current of 7 ma was applied for 16 hours. To prevent the precipitation of fibrinogen, the strips were wetted with 50 ml of buffer containing 10 mg of heparin. After completing the run, the strips were dried at 125°C for 30 minutes, stained with bromphenol blue for 6 hours, rinsed 3 times in 5% acetic acid and dried at 125°C for 15 minutes. Finally, they were exposed to ammonia vapor and read in a recording densitometer. A number of strips with maternal or fetal samples were stained also for plasma polysaccharides(4).

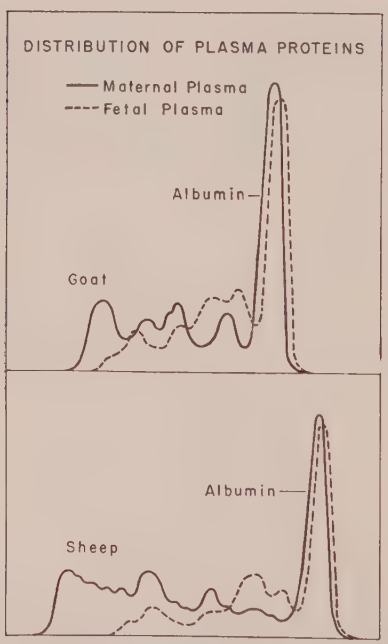

FIG. 1. Electrophoretic patterns of maternal and fetal blood plasma proteins. Goat fetus 125 days old, sheep fetus 128 days old.

Results. An illustration of differences in fetal and maternal patterns of plasma proteins can be seen in Fig. 1. The γ-globulin peak is missing in both fetal sheep and goats, probably because the multicellular barrier of the syndesmochorial placenta blocks the transfer of these macromolecules, and the fetal organism is not able to synthesize them(2). Human fetal plasma has been reported to contain some γ-globulins, as the unicellular hemochorial type of human placenta allows passage of some proteins(5). In the area of the albumin-bordering single peak of α_1-globulins in maternal plasma, the fetal pattern exhibits 2 peaks.

The changes in the quantitative aspects of individual fetal plasma proteins, as they are related to the age of the fetus, are reported in Table I. These data are based on determinations on single animals for each date and are, therefore, subject to individual variations. They show, however, the direction of changes quite clearly. In agreement with previous reports(6), the percentage of total plasma proteins tended to increase with the progressing fetal age. This tendency, because of the small number of samples and individual variations, was somewhat obscured in the sheep group. In both sheep and goats the percentage of plasma albumin rose with age of the fetus. Regarding the two peaks next to

TABLE I. Plasma Protein Fractions in Fetal Goat and Sheep.

Age of fetus, days	Total protein, g %	Albumin, %	Fetuin, %	Globulins (%) α_1	α_2	β
			Goats			
64	2.8	23.0	28.9	17.8	14.8	15.5
85	3.3	34.9	25.9	16.3	12.7	10.2
103	3.0	38.1	19.3	17.7	11.2	13.7
110	3.5	38.2	18.0	19.1	10.7	14.0
113	4.0	40.5	17.8	20.3	11.7	9.7
119	4.0	39.4	15.4	16.8	12.0	16.4
127	3.6	43.1	16.0	15.3	14.2	11.4
131	3.7	43.9	14.7	19.2	10.6	11.6
			Sheep			
49		20.2	13.1	46.4	8.3	11.9
57		20.2	7.5	44.7	10.6	17.0
64	2.5	28.0	9.0	42.0	12.0	9.0
128	3.5	39.6	8.3	26.6	8.9	16.6
138	3.7	42.1	6.2	19.4	8.1	24.2

albumin, neither superimposing of the fetal and maternal protein patterns, nor chemical analysis of the 2 bands for polysaccharides reported to contain fetuin(7) were of help in disclosing which of the 2 peaks was fetuin. Both fractions showed a relatively high content of polysaccharides; higher than the corresponding maternal α_1-globulins. Taking an additional reported property of fetuin into account, *e.g.*, the decrease in concentration with the age of fetus(1,8), one can assume that in goats the peak next to the albumin is fetuin. In sheep the conditions are somewhat more complicated, as both the first and especially the second peak decrease with age.

As to the remaining plasma proteins, the α_1-, α_2-, and β-globulins of the goat did not seem to be influenced by the age of the fetus. In the fetal sheep the concentration of α_2-globulins did not show any significant correlation with age; the percentage of the β-globulins tended to increase, but as mentioned before, the number of samples is too small to allow a definite conclusion.

The age-correlated decrease and the relatively high content of polysaccharides in fetuin reminds one of Wharton jelly, a substance of fetal cord rich in mucopolysaccharides(9), which reportedly also decreases in amount with the growing fetus(10). The similar fate and proximity suggest a possible relationship.

Summary. Electrophoretic separation of fetal and maternal plasma proteins in goats and sheep revealed that the fetal plasma lacks γ-globulins and exhibits an additional peak in the area of α_1-globulins. This peak probably corresponds to fetuin. Quantitative conditions of fetal plasma proteins seem to depend largely on age of the fetus. In goats, the percentage of total plasma proteins and of plasma albumin increases; percentage of fetuin decreases with the progressing age of the fetus. The remaining plasma proteins do not seem to be affected. In fetal sheep the percentages of total plasma proteins, plasma albumin and probably of β-globulins increase; levels of fetuin and α_1-globulins decrease with age. The percentage of α_2-globulins remains unaffected.

We wish to thank Miss Patricia Daniels for technical assistance.

1. Pedersen, K. O., *Nature,* 1944, v154, 575.
2. Hansen, R. G., Phillips, P. H., *J. Biol. Chem.,* 1947, v171, 223.
3. Rutqvist, L., *Am. J. Vet. Res.,* 1958, v19, 25.
4. Köiw, E., *Scand. J. Clin. Lab. Inv.,* 1952, v4, 244.
5. Dancis, J., Braverman, N., Lind, J., *J. Clin. Inv.,* 1957, v36, 398.
6. Meschia, G., *Am. J. Physiol.,* 1955, v181, 1.
7. Deutsch, H. F., *Fed. Proc.,* 1953, v12, 196.
8. Bergstrand, C. G., Czar, B., *Scand. J. Clin. Lab. Inv.,* 1957, v9, 277.
9. Romanini, M. G., *Acta Anat.* (Basel), 1951, v13, 257.
10. Barcroft, J., Barron, D. H., *Anat. Rec.,* 1946, v94, 569.

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Infectious Bovine Rhinotracheitis (IBR) IV. Cytological Changes in Infected Bovine Kidney and HeLa Cultures. (24133)

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Specific intra-nuclear inclusions were observed in routine celloidin preparations of infected HeLa and primary bovine kidney culture monolayers during adaptation to HeLa cells of infectious bovine rhinotracheitis (IBR) virus(1). Since specific localization of increased amounts of desoxyribonucleic acid (DNA)(2) and of other constituents(3) often has resulted from cellular changes produced by infection with other viruses, more detailed cytologic observations of the effects of IBR virus were undertaken. The morphological and chemical effects of IBR virus on cells of an artificial host (HeLa) and of a

natural host such as primary bovine kidney (PBK) were compared. This report presents a comparison of the form and staining properties of HeLa and PBK inclusions following use of Feulgen technic.

Materials and methods. HeLa and PBK cultures were prepared and infected as previously described(1) except that cells were grown on microslips in Leighton tubes. In brief, HeLa cells were grown in Eagle's basal medium(4) with 20% inactivated horse serum. Trypsinized bovine embryo kidney cells, prepared by the method of Dulbecco and Vogt(5), were grown in Earle's basal medium(6) with 20% inactivated cow serum and lactalbumin hydrolysate(7). After monolayers had been established (3-4 days), the growth medium in both HeLa and PBK cultures was replaced by the same control or infected maintenance medium. Control media consisted of supernate from non-infected HeLa cultures diluted (10%) with Ginsberg medium(8). Infectious media consisted of supernate from IBR-infected HeLa cultures diluted (10%) with Ginsberg medium. Examination of both control and infected cultures for signs of cellular degeneration was made daily with a 10x objective. When infected HeLa cultures showed scattered areas of degenerated cells, all cultures were fixed, to insure availability of sufficient cells of both types with inclusions. Waiting for extensive sloughing of HeLa cells would have allowed the PBK cells to degenerate almost completely and to disappear from the glass. The Feulgen technic was based on the method of Gardikas and Israels(9), with substitution of the following wet fix: methyl alcohol 15 ml, distilled water 10 ml, glacial acetic acid 0.25 ml, 40%-formalin 1 ml. For hematoxylin-eosin (H & E) staining, Bouin fixative was used.

Results. H & E staining demonstrated in both types of infected cells a characteristic pattern of nuclear degeneration not seen in control cultures of either type. Inclusions appeared in infected cells before any striking cytoplasmic change could be observed, and resembled in form and general staining properties the H & E-stained primary bovine kidney and human amnion cells reported by Cheatham *et al.*(10). In brief, the normally dispersed, acidophilic, fine nuclear substance condensed and was left surrounded by a fairly uniform clear zone. The nucleoli were reduced in number and displaced toward the periphery of the nucleus. Accompanying margination of the chromatin at the nuclear membrane, resulted in intensified staining with basic dye components, which sharply delineated the nucleus.

The similarity of PBK and HeLa inclusions demonstrated by H & E staining also was demonstrated by use of Feulgen technic. After fixation, the microslips with control and infected cultures of both cell types were removed from their tubes and transferred to the same carrier, making it possible to subject all culture slips simultaneously to hydrolysis, treatment with Schiff's reagent, and counterstaining with Fast Green. In some preparations of both control and infected PBK and HeLa cultures, the Fast Green counterstain was omitted.

In inclusions of both PBK and HeLa nuclei, the absence of DNA was invariably demonstrated, regardless of staining method used or duration of infectivity. In both PBK (Fig. 2) and HeLa (Fig. 4) cultures, the Feulgen-negative inclusions stained readily with Fast Green. Nucleoli of both cell types stained deep green, in sharp contrast to the less intense green of the condensed inclusion and the nuclear membrane. The marginated chromatin of the latter was strongly Feulgen-positive. A decrease of DNA from the nuclei of both cell types also accompanied proliferation of the HeLa-strain IBR virus. This reduction of Feulgen-positive material was evident even at the periphery of the nucleus where the chromatin had marginated, as evidenced by less intense staining.

Use of the Feulgen technic also disclosed two interesting differences between the HeLa and the PBK cells in control cultures. (A) PBK cytoplasm showed relatively less affinity to Fast Green, taking approximately 10 times as long to stain as HeLa. (B) Without counterstaining, the strikingly greater concentration of Feulgen-positive material observed in the smaller PBK nuclei suggested that they contained relatively more DNA

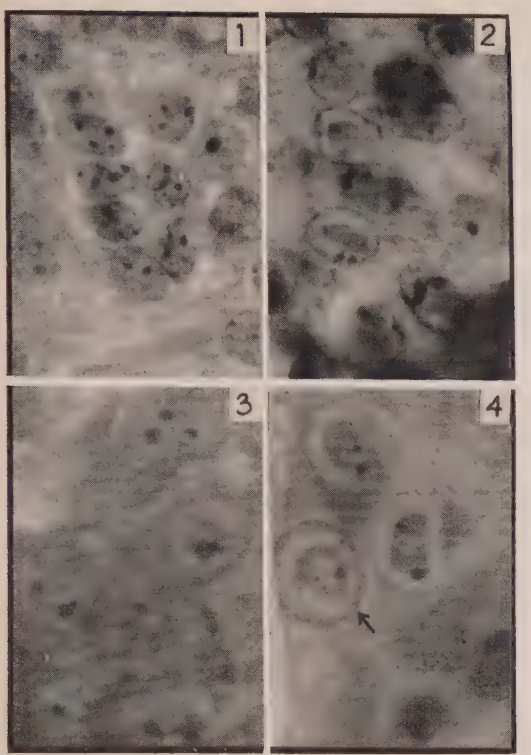

FIG. 1. Uninfected bovine kidney monolayer. Chromatin, acidophilic nuclear substance, and nucleoli are uniformly distributed.

FIG. 2. IBR-infected bovine kidney monolayer with majority of nuclei showing "inclusions."

FIG. 3. Uninfected HeLa monolayer with normal complement of nucleoli, dispersed chromatin and acidophilic element. Note that HeLa nuclei tend to be more obscured by Fast-Green-stained cytoplasm than bovine nuclei in Fig. 1.

FIG. 4. Typical IBR-infected HeLa nuclei, with one (arrow) demonstrating breakdown of the nucleoli into smaller deeply stained (Fast Green) particles.

(All fields are of Feulgen-Fast-Green-stained preparations photographed at ×588.)

than the HeLa nuclei.

Discussion. Although HeLa and PBK cells differed somewhat with respect to size, staining properties and rate of virus-induced degeneration, no difference in morphology or staining properties of the inclusions was demonstrated by infecting the 2 cell types with the same strain of IBR virus in the same maintenance medium. Infection was followed by loss of DNA in both HeLa and PBK cells, and no Feulgen-positive inclusion material could be demonstrated, either during early stages of infection when only a few isolated cells showed inclusions, or in later stages when almost all remaining cells contained well formed inclusions. In this way IBR virus differs from those viruses which cause increased intranuclear DNA inclusion as infection progresses, as is seen in tissues infected with canine hepatitis(11) and in HeLa cells infected with adenovirus(2).

Summary. In both bovine kidney and HeLa cultures, extensive focal nuclear degeneration followed inoculation with a HeLa-adapted IBR virus strain. The pattern of nuclear and cytoplasmic degeneration was similar for the native host (PBK) and the HeLa cell. In cells of both types, nuclear inclusions were Feulgen-negative, and morphologic nuclear changes were accompanied by a reduction of DNA.

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1. Cabasso, V. J., Brown, R. G., Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 471.

2. Bloch, D., Morgan, C., Godman, G., Howe, C., Rose, H., *J. Biophys. and Biochem. Cytol.*, 1957, v3, 1.

3. Orsi, E. V., Love, L., Koprowski, H., *Cancer Res.*, 1957, v17, 306.

4. Eagle, H. J., *J. Exp. Med.*, 1955, v102, 595.

5. Dulbecco, R., Vogt, M., *ibid.*, 1954, v99, 167.

6. Earle, W. R., *J. Nat. Cancer Inst.*, 1943, v4, 165.

7. Melnick, J. L., Riordan, J. T., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 208.

8. Ginsberg, H. S., Gold, E., Jordan, W. S., Jr., *ibid.*, 1955, v89, 66.

9. Gardikas, C., Israels, M. C. G., *J. Clin. Path.*, 1948, v1, 226.

10. Cheatham, W. J., Crandell, R. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 536.

11. Ohno, S., Kinosita, R., *Exp. Cell Res.*, 1954, v7, 578.

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Transfer of Acquired Tolerance to Skin Homografts in Mice.* (24134)

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The induction of acquired tolerance to homotransplantation of several kinds of tissues in animals of different species treated at birth with living foreign spleen cells has been firmly established(1,2,3,4,5). However not much is known as to the mechanisms involved in the induction or maintenance of this immunological phenomenon. Based on our observations on tolerance in mice to homologous tumors inoculated during their neonatal period, it was tentatively postulated that persistence of immunological tolerance might be dependent on presence of foreign living cells in the tolerant individual(6). These observations suggested that one way to test this hypothesis would be to transfer tolerance by way of spleen cells taken from a tolerant animal, by injecting these cells into newborn individuals of the same strain. Specifically, the purpose of these experiments was to ascertain whether or not acquired tolerance induced in "B" strain individuals to "A" strain tissues could be transferred to other animals of the "B" strain by intravenous injection at birth of spleen cells taken from "B" adult donors previously made tolerant to "A" strain tissues.

Method. Inbred mice of the ZBC‖ and Ce stocks were used. ZBC animals are backcross hybrids to the Z(C3H) strain and genetically non-related to those of the Ce strain. In a first step, tolerance to Ce skin homograft was induced in ZBC animals by intravenous injection at birth (not later than 24 hours) of 2-4 million living spleen cells taken from adult Ce donors. The method of preparation of the spleen cell suspension and that of intravenous injection has been described previously(5,7,8). When treated animals were approximately 2 months of age or older, they received a full thickness skin graft taken from adult Ce donors. Technic for skin graft and criteria used to judge its success or failure have also been described(5). Treated ZBC animals accepting the skin graft for no less than 2 months were considered as tolerant to Ce tissue and used as donors of the spleen to be used in second part of experiment. This consisted essentially in removal of spleen from ZBC animals which were tolerant to Ce. These spleen cells were then prepared as a new spleen cell suspension which was injected into newborn animals of the ZBC strain. The preparation of these cells and the injection were performed as in the first phase of the experiment. When this second group of animals were approximately 2 months old they were tested for tolerance to skin grafts taken from adult Ce donors. A group of non-treated ZBC animals also received a Ce homologous skin graft and served as controls.

Results are listed in the Table. The incidence of successful Ce skin grafts in ZBC animals injected at birth with living spleen cells taken from Ce donors was 94% of a total of 35 mice tested. Similarly the incidence of successful Ce skin grafts in ZBC animals injected at birth with living spleen cells taken from ZBC donors made previously tolerant to Ce tissues was 83% from a total of 30 animals. The difference between this incidence and that in the former group is not statis-

TABLE I. Transfer of Tolerance to Skin Homografts in Mice.

Spleen donor strain	Recipient strain	Skin grafted	No. of mice accepting skin graft
			%
Ce	ZBC	Ce	33/35 (94)
ZBC tol. Ce	"	"	25/30 (83)
	"	"	0/66 (0)

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‡ Am. Cancer Soc. Scholar.

§ Am. Legion Heart Memorial Professor of Pediatrics.

‖ ZBC hybrids are obtained as follows:
A ♀ x Z(C3H) ♂ = AZ F_1 ♀ x Z(C3H) ♂ = ZBC

tically significant. In contradistinction the 66 non-treated ZBC controls all rejected the Ce homologous skin as would be predicted from their genetic differences.

Discussion. Our results agree with the hypothesis which provoked this study. In fact, it was originally postulated that if the state of tolerance requires the presence of living foreign cells in the host it might be possible to transfer this tolerance to another individual of the same strain by injecting at birth the spleen containing the homologous foreign cells necessary to induce this phenomenon. Our observations using the donor-host strain combination Ce⟶ZBC indicate that this has been accomplished, since by treating newborn ZBC animals with living spleen cells taken from ZBC animals tolerant to Ce donor, tolerance to homologous Ce skin was demonstrated.

This observation also agrees with that of Billingham, Brent and Medawar(2) who showed that spleen tissue from tolerant animals contains antigenically active material from the donor strain. Our findings however extend those of the British workers and indicate that in the tolerant host all of the transplantation antigens presumably in the form of many intact cells derived from the original donor are present in the reticuloendothelial tissues of the tolerant host.

Summary. Newborn mice of the ZBC strain injected intravenously with living spleen cells taken from donors of the same strain in which tolerance to Ce tissues was previously induced, became tolerant to this strain and accepted homologous skin grafts from Ce donors. This would indicate that acquired tolerance can be transferred to an isologous individual at birth by the intravenous injection of spleen cells taken from a tolerant donor.

1. Billingham, R. E., Brent, L., Medawar, P. B., *Nature,* 1953, v172, 603.

2. Billingham, R. E., *Phil. Trans. Roy. Soc. London,* 1956, v239B, 357.

3. Woodruff, M. F. A., Simpson, L. O., *Brit. J. Exp. Path.,* 1955, v36, 494.

4. Egdahl, R. H., Varco, R. L., *Surg. Forum,* 1957, v8, 589.

5. Martinez, C., Smith, J. M., Aust, J. B., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 736.

6. Aust, J. B., Martinez, C., Good, R. A., *Proc. Am. Assn. Cancer Research,* 1957, v2, 185.

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Hypothalamus and Somatotrophic Hormone Release. (24135)

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Hypothalamic control of somatotrophic hormone (STH) secretion, though not yet clearly demonstrated, may be assumed from data in the literature. Hetherington *et al.*(1), Bogdanove *et al.*(2), and Endröczi *et al.*(3) showed that rats with hypothalamic lesions frequently fail to grow; retardation of body growth has also been reported in dogs in which anterior hypothalamic lesions had been performed early in their life(3). Typical disturbances of carbohydrate metabolism induced by hypophysectomy and by STH deficiency have been found also after hypothalamic lesions involving the region of paraventricular nuclei(4); Spirtos *et al.*(5) observed that lesion-bearing rats often exhibit increased hypoglycemic response to insulin, which can be reduced by STH administration. It is generally agreed that marked retardation of body growth follows transplantation of the pituitary far from the sella turcica (6,7); it is noteworthy that both hypothalamic lesions(2) and hypophyseal transplantation(7) induce disappearance of the STH-producing eosinophil cells of the anterior pituitary. The mechanism by which hypothalamic stimuli may reach the glandular cells of the anterior lobe of the pituitary gland

and regulate release of trophic hormones has been widely discussed(6); results of anatomical and physiological studies support the view that the hypophyseal portal vessels regulate anterior pituitary functions by transmitting an hypothalamic humoral substance(6). Several suggestions have been put forward as to the nature of this hypothalamic neurohumor; attention was called to a possible relationship between antidiuretic hormone (ADH) of the hypothalamic-posthypophyseal system, and release of ACTH(8). This view was mainly based on the demonstration that extracts containing ADH have an ACTH releasing effect in normal animals(9,10), in hypophysectomized animals bearing a functional pituitary graft in the anterior chamber of the eye(7) and in rats with hypothalamic lesions (11). Recently ACTH-releasing activity of posterior pituitary preparations has been confirmed in an *in vitro* system, in this case ascribed to the presence of a contaminant(12, 13). The experiments here reported deal with the possibility of inducing somatotrophic hormone release (STH) by means of the same mediator which is effective in inducing ACTH release.

Methods. Normal and hypophysectomized Sprague-Dawley rats, of both sexes, weighing 60 g were used. They were given basal diet and tap water; hypophysectomized rats received an additional 5% glucose in drinking water. Hypophysectomy was performed by standard parapharyngeal approach. Preparations of whole posterior pituitary lobe extract (PPLE) (Postipofisan, Richter) and purified antidiuretic (Pitressin, Parke-Davis) and oxytocic (Pitocin, Parke-Davis) hormones were used.* These hormones were given by daily intraperitoneal injections (0.5 U/rat dissolved in 0.5 ml of 0.9% NaCl solution) for 10 days; control rats were injected daily with 0.5 ml of 0.9% saline solution. Three groups of normal male rats were given intraperitoneally an STH preparation (Armour, 80 U/mg) in daily doses of 25, 50, and 100 μg/rat, for 4 days. All animals were killed 24 hours after last injection; treatment of hypophysectomized rats started 10 days after operation. The tibias of animals were then prepared for measurement of width of proximal epiphyseal cartilage according to Greenspan's *et al.* standard procedure(14). Only female rats have been used in the previous STH studies; however Geschwind *et al.*(15) established that male rats are sensitive to STH.

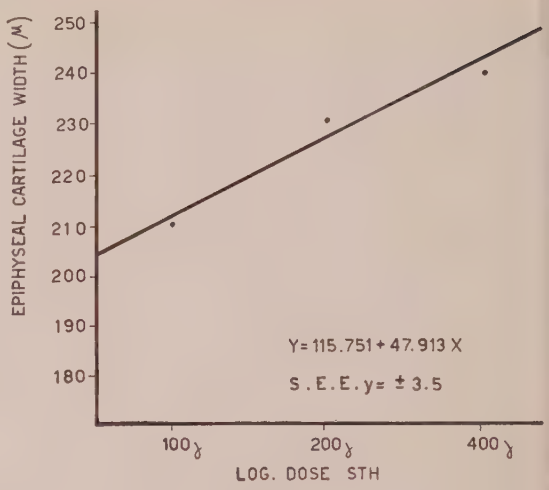

FIG. 1. Effect of exogenous STH on tibia cartilage width of normal male rats.

Results. Fig. 1 shows that an enlargement of the tibia cartilage width may be induced in normal male rats by injecting different doses of STH; the curve, and its regression equation, show that a linear log-dose relationship may be obtained. The results summarized in Table I show that chronic treatment with

TABLE I. Effect of Continuous Injections of Different Posterior Pituitary Preparations on Epiphyseal Cartilage Width of Normal and Hypophysectomized, Male and Female Rats.

Treatment	Sex of rats	No. of rats	Tibia cartilage width (μ) Normal rats	Hypophysectomized rats
.9% NaCl	♂	12	184 ± 8.3	117 ± 11.6
PPLE	♂	13	221 ± 8.1*	113 ± 3.8
.9% NaCl	♂	10	204 ± 3.3	112 ± 15.9
Pitressin	♂	8	236 ± 5.6*	107 ± 17.5
Pitocin	♂	8	199 ± 3.9	
.9% NaCl	♀	8	203 ± 5.8	
PPLE	♀	7	233 ± 6.8†	

* Differs significantly from controls ($P < .001$).
† *Idem* ($.005 > P < .01$).

* We are indebted to Dr. Z. Koreny of Richter Laboratories, and to Dr. D. A. McGinty of Parke-Davis and Co. for generous supplies of posterior pituitary preparations.

PPLE and with Pitressin induces significant enlargement of epiphyseal cartilage width in normal male rats ($P<0.001$); PPLE induces an increase in tibia cartilage width also in normal female rats ($P<0.01$); Pitocin is ineffective in normal male rats. In the hypophysectomized animals PPLE and Pitressin do not produce significant modification of the epiphyseal cartilages.

Discussion. Our experiments clearly demonstrate that different posterior pituitary preparations containing ADH may stimulate proliferation of tibia epiphyseal cartilage in normal rats of both sexes; lack of activity of these same preparations in hypophysectomized animals seem to rule out the possibility of an STH contamination of the extracts used, and to suggest that the stimulus on the cartilage plate is mediated through release of an adenohypophyseal principle; these experiments also suggest that STH may be the hormone involved.

A slight enlargement of the cartilage width may be produced by thyroxine and testosterone(15,16); it is then conceivable that TSH and ICSH through activation of their target glands may act as stimulators of the epiphyseal cartilage plate. A TSH-releasing activity of posterior pituitary preparations(17,18) may suggest release of TSH as a possible explanation of the results here described; however, Geschwind *et al.*(15) found that very large doses of exogenous thyrotrophic hormone are required to duplicate the effects of small amounts of STH on the tibia test. As to the role of ICSH, very little is known about the action of posterior pituitary principles on release of gonadotrophic hormones. Pitocin could be the stimulus for the pituitary-gonadal system, as judged by output of urinary gonadotrophin and fractionation of 17-ketosteroids(19,20). But according to our results Pitocin is not active on the cartilage plate; moreover liberation of ICSH after giving posterior pituitary preparations could play a role only in male animals(15), whereas their effectiveness on the cartilage width occurs in both sexes. Prolactin might also duplicate the effects of STH(21-23) as Marx *et al.*(16) reported a slight enlargement of the epiphyseal cartilage after giving prolactin but Geschwind *et al.*(15) demonstrated that this effect was sex-dependent, because of its presence only in male rats. In the present experiments the effect of posterior pituitary preparations on the cartilage plate was evident both in female and in male rats; moreover should this effect be due to prolactin, it would also be present after giving Pitocin, which is very active in promoting prolactin release(23,24).

Summary. 1) Chronic treatment with posterior pituitary preparations containing ADH induces significant enlargement of tibia cartilage width in normal male and female rats; oxytocic hormone is ineffective in normal animals. 2) In hypophysectomized animals posterior pituitary hormones do not produce modification of the epiphyseal cartilages.

1. Hetherington, A. W., Ranson, S. W., *Endocrinol.,* 1942, v31, 30.

2. Bogdanove, E. M., Lipner, H. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 410.

3. Endröczi, E., Kovacs, S., Szalay, G., *Endokrinologie,* 1957, v34, 168.

4. Harris, G. W., *Physiol. Rev.,* 1948, v28, 139.

5. Spirtos, B. N., Ingram, W. R., Bogdanove, E. M., Halmi, N. S., *J. Clin. Endocrin.,* 1954, v14, 790.

6. Harris, G. W., *Neural Control of the Pituitary Gland,* Edward Arnold Ltd., London, 1955.

7. Martini, L., De Poli, A., *J. Endocrin.,* 1956, v13, 229.

8. Martini, L., *Pathophysiologia Diencephalica,* Springer Verlag, Wien, 1958.

9. Martini, L., Morpurgo, C., *Nature, Lond.,* 1955, v175, 1127.

10. Martini, L., De Poli, A., Curri, S., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 490.

11. McCann, S. M., Brobeck, J. R., *ibid.,* 1954, v87, 318.

12. Saffran, M., Schally, A. V., Benfey, B. G., *Endocrinol.,* 1955, v57, 439.

13. Guillemin, R., Hearn, W. R., Cheek, W. R., Housholder, D. E., *ibid.,* 1957, v60, 488.

14. Greenspan, F. S., Li, C. H., Simpson, M. E., Evans, H. M., *ibid.,* 1949, v45, 455.

15. Geschwind, I. I., Li, C. H., The Hypophyseal Growth Hormone, Blakiston Division, N. Y., 1955, p28.

16. Marx, W., Simpson, M. E., Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 250.

17. Dubreuil, R., Martini, L., XX Internat. Physiol. Congress, Abst., 1956, p257.

18. Bottari, P., *Ciba Foundation Colloquia on Endocrinology,* 1957, v52, 11.

19. Martini, L., Mira, L., Pecile, A., Saito, S., III Acta Endocrinol. Congress, Leiden, 1958.

20. Shibusawa, K., Saito, S., Fukuda, M., Kawai, T., Yoshimura, F., *Endocr. Jap.,* 1955, v2, 47.

21. Scooley, J. P., Riddle, O., Bates, R. W., *Am. J. Anat.,* 1941, v69, 123.

22. Miller, R. A., Riddle, O., *Endocrinol.,* 1943, v32, 463.

23. Furth, J., Clifton, K. H., Gadsden, E. L., Buffett, R. F., *Cancer Res.,* 1956, v16, 608.

24. Benson, G. K., Folley, S. J., *J. Endocrin.,* 1957, v16, 189.

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Increased Creatine and Creatinine Excretion after 17α-Ethyl-19-Nortestosterone.* (24136)

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Among biological processes, the rate of creatine synthesis in mammals is remarkable for its constancy. Creatine is formed in the liver by methylation of guanidinoacetic acid (1). It then diffuses freely throughout the body; concentration of free creatine in various cells and body fluids is approximately equal. Creatine, particularly in muscle, is phosphorylated to form the labile phosphocreatine, thus concentration of total creatine in cells is higher than that of free creatine(2). There is a renal threshold for creatine(3), and under normal circumstances very little appears in the urine. Creatinine, the end product of creatine metabolism, arises from creatine or phosphocreatine by non-enzymatic dehydration(4). At closely regulated pH and temperature of body, its rate of formation appears to depend only on concentration of creatine. In man, about 1.64% of the creatine body pool is converted to creatinine each day (5). Creatinine does not enter into any metabolic reactions and ultimately is completely excreted in the urine. While fluctuations in excretion in consecutive short term collections are known to occur, creatinine content of successive 24 hour collections is quite uniform (6,7). Since a steady state of formation and excretion is established, even in illness, the measure of combined creatine and creatinine chromogen in urine is also a measure of creatine synthesis. The factors which regulate rate of synthesis of creatine are not well known. Changes in peripheral requirements for creatine do not seem to modify its production(8). However, Wilkins, Fleischmann and Howard(9) found that methyltestosterone, but not testosterone, increased markedly the formation of creatine. This communication reports a similar, perhaps more profound increase in creatine synthesis as judged by excretion of total creatinine chromogen after oral administration of 17α-ethyl-19-nortestosterone.

Materials and methods. Six apparently healthy adults were given 30 mg 17α-ethyl-19-nortestosterone (Nilevar) daily by mouth. A 24 hour urine collection was obtained prior to and after 6 weeks of drug administration for determination of creatine, creatinine, total creatinine chromogen and uric acid. Subjects were placed on a creatine-free diet for 4 days before urine collections were obtained. Creatine was determined by the method of Anderson, *et al.*(10), creatinine was measured by the method of Owen, *et al.*(11), and uric acid was determined by the method of Brown(12).

Results. Increase in excretion of creatine and total creatinine chromogen was observed in all subjects. Mean daily excretion of creatine was 81.4 mg before and 473.3 mg after 6 weeks of drug with a mean increase in creatine excretion of 393.7 mg. Mean daily excretion of total creatinine chromogen was 1.58 g before and 2.35 g after 6 weeks of drug

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TABLE I. Urinary Creatine and Creatinine Excretion before and after 6 Weeks of 30 mg. 17 α-methyl-19-nortestosterone daily.

Age, sex, wt (kg) of subject	Creatine, mg/24 hr	Creatinine, g/24 hr	Total creatine chromogen, g/24 hr	Uric acid, g/24 hr
36, ♀, 59.1	27.8	1.10	1.13	.43
	612.4	*1.20*	*1.82*	*.42*
30, ♂, 82.7	98.8	1.85	1.95	.66
	612.0	*3.27*	*3.89*	*.81*
27, ♂, 74.6	74.2	1.65	1.72	.46
	122.8	*2.12*	*2.25*	*.48*
25, ♂, 77.3	103.0	1.78	1.88	.63
	422.2	*2.01*	*2.43*	*.75*
32, ♂, 78.2	129.6	1.76	1.88	.59
	153.3	*1.93*	*2.08*	*.59*
27, ♀, 66.0	55.2	.87	.93	.20
	917.0	*.72*	*1.62*	*.36*
Mean increment	393.7	.36	.77	.07

Values after drug are in italics.

with a mean increment of 0.77 g. The results are given in Table I.

It is difficult to reconcile these results with an effect of 17α-ethyl-19-nortestosterone on the kidney. While it is conceivable that increase in filtration of creatinine might occur or change in renal "threshold" for creatine might occur, a new steady state of synthesis and excretion would be quickly established and excretion of total creatinine chromogen would be again limited by, and a reflection of, the rate of creatine synthesis.

The mechanism whereby 17α-ethyl-19-nortestosterone acts to increase excretion of creatine and creatinine, and therefore synthesis of creatine, is not clear. Both this drug and methyltestosterone, which produces creatinuria, cause a positive nitrogen balance and an increase in muscle mass and tone(13). Perhaps the alteration of creatine metabolism which has been observed is a part of the general anabolic effects of these steroids. Increasing creatine synthesis may be a generic effect of 17-alkylated androgens.

Summary. A mean increase of 393 mg in daily excretion of creatine and an increase of 0.77 g in daily excretion of total creatinine chromogen has been observed in 6 subjects after oral administration of 30 mg 17α-ethyl-19-nortestosterone daily for 6 weeks.

1. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., Simmonds, S., *J. Biol. Chem.,* 1941, v140, 625.

2. Ennor, A. H., Rosenberg, H., *Biochem. J.,* 1952, v51, 606.

3. Zierler, K. L., Folk, B. P., Eyzaguirre, C., Jarcho, L. W., Grob, D. W., Lilienthal, J. L., Jr., *Ann. N. Y. Acad. Sci.,* 1949, v52, 180.

4. Bloch, K., Schoenheimer, R., Rittenberg, D., *J. Biol. Chem.,* 1941, v138, 155.

5. Hoberman, H. D., Sims, E. A. H., Peters, J. H., *ibid.,* 1948, v172, 45.

6. Shaffer, P. A., *Am. J. Physiol.,* 1908, v23, 1.

7. Vestergaard, P., Leverett, R., *J. Lab. Clin. Med.,* 1958, v51, 211.

8. Zierler, K. L., Folk, B. P., Magladery, J. W., and Lilienthal, J. L., Jr., *Bull. Johns Hopkins Hosp.,* 1949, v85, 370.

9. Wilkins, L., Fleischmann, W., Howard, J. E., *ibid.,* 1941, v69, 493.

10. Anderson, D. R., Williams, C. R., Krise, G. M., Dowben, R. M., *Biochem. J.,* 1957, v67, 258.

11. Owen, J. A., Iggo, B., Scandrett, F. J., Stewart, C. P., *ibid.,* 1954, v58, 426.

12. Brown, H., *J. Biol. Chem.,* 1945, v158, 601.

13. McSwiney, R. R., Prunty, F. T. G., *J. Endocrin.,* 1957, v16, 28.

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Effect of Dinitrophenol on Lens of Chick Embryo.* (24137)

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Horner(1) reported that therapeutic use of 2,4-dinitrophenol (DNP) for control of obesity had resulted in cataracts in a small number of patients. Robbins(2) produced cataracts in the chick by addition of 0.25% DNP to the diet. Occurrence of this lesion was not prevented by addition of riboflavin to the diet. Buschke(3) showed that a threshold dose of 0.06 mM of DNP/kilo body weight produced an opacity in the lens of the chick when administered either by stomach tube or intramuscularly. Bettman(4) produced cataracts in chicks and rabbits by direct injection of DNP into the anterior chamber of the eye. Rabbits, however, did not develop cataracts when fed DNP. This study was initiated to determine the effect of DNP on the lens of the chick embryo.

Method. White Leghorn X New Hampshire eggs were injected with a solution of DNP in physiological saline prior to incubation and on fifth, eighth and fifteenth day of the 21-day incubation period. A small area over the air cell of the egg was painted with tincture of merthiolate and a small hole drilled through the shell. A 23 gauge needle was used for injection of DNP solution. The hole was then sealed with collodion after injection. The eggs were incubated in a standard commercial incubator. The eggs were candled daily and dead embryos removed for gross study and an estimation of time of death. Embryos which failed to hatch were treated similarly. The eyes of live embryos and chicks that hatched were examined for lenticular changes with a Bausch and Lomb Ophthalmoscope equipped with May-type optics. The number of embryos, amount of DNP and time of injection are shown in Tables I and II.

Results. Eighty eggs were injected with 1-25 μg of DNP prior to incubation (Table I). The mortality was high on third day of incubation and progressively increased with increasing concentrations of DNP injected. Surviving embryos appeared normal when hatched. The live-unhatched embryos exhibited an edematous area at the back of the neck similar to that described by Ferguson *et al.*(5) in Vit. E-deficient turkey embryos. No lens changes were observed in the chicks or live-unhatched embryos. There is a normal mortality peak at the third day of incubation of chicken eggs(6). This peak is amplified in deficiencies of Vit. E, biotin or riboflavin. Needham(7) advanced evidence suggesting a change in oxidative metabolism of carbohydrates occurring at third day which may be responsible for the normal mortality peak. It is possible that DNP interferes with this change.

One hundred and seventy-three fertile eggs were injected with 25-200 μg of DNP on the fifth day of incubation (Table II). Embryonic mortality after injection increased with increasing levels of DNP employed with all embryos killed by the 200 μg level (Table II). Cataracts were not observed in samples examined 20 hours after injection or embryos examined on 19th day.

TABLE I. Effect of Injecting DNP Prior to Incubation, 20 Eggs/Series.

Treatment	No. of fertile eggs	No. dead at 3 days	No. chicks hatched	% hatch of fertile eggs
Uninj.	18	1	17	94.4
Drilled, uninj.	18	2	14	77.8
.1 ml physiol. saline	18	3	12	66.7
1 μg DNP	17	10	5	29.4
5 " "	15	10	4	26.7
10 " "	16	11	3	18.8
25 " "	14	11	1	7.1

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† Public Health Service Research Fellow, Nat. Inst. of Neurological Diseases and Blindness.

TABLE II. Effect of DNP Injected after 5, 8 and 15 Days of Incubation.

Day of inj.	Treatment, μg DNP	No. fertile eggs	20 hr after inj. No. removed for observation	20 hr after inj. No. with cataracts	20 hr after inj. No. dead embryos	Unhatched embryos No.	Unhatched embryos No. with cataracts*	Hatched chicks No.	Hatched chicks No. with cataracts*
5	Saline control	19	4	0	1	1	0	13	0
	25	35	4	0	4	4	0	23	0
	30	14	4	0	9				
	40	14	4	0	9				
	50	14	4	0	7				
	60	14	4	0	7				
	70	14	4	0	10				
	80	14	4	0	9				
	100	29	6	0	22				
	200	25	5	0	20				
8	Saline control	20	6	0	1	2	0	11	0
	25	48	6	0	2	2	0	38	0
	100	36	6	0	1	5	0	24	0
	200	20	8	8	2	2	1	8	3
	300	20	6	6	8	2	2	4	2
	400	20	6	6	11	1	1	2	1
	500	20	6	6	8	2	2	4	4
15	Saline control	20	4	0	0	1	0	15	0
	100	30	5	0	1	3	0	21	0
	750	47	5	5	20†	2	2†	20	0

* Cataract in both eyes.
† Opacities observed in all cases may have been post-mortem changes.

One hundred sixty-four eggs were injected with 25-500 μg DNP after 8 days incubation. Cataracts were produced only in embryos from groups injected with 200-500 μg (Table II). Approximately one-third of the lens area was opaque when viewed from the anterior aspect. Opacity was situated in the center of the lens and proceeded posteriorly to the central cortical area. Seven live embryos which failed to hatch had cataracts and exhibited an extensive edematous area of gelatinous nature at back of neck, not observed in the control groups. This condition is similar to that described for Vit. E-deficient turkey poults(5). In a few cases the edema was so extensive that the vertebrae could be easily seen. A crude measurement was made of the cataract with the grid system of the ophthalmoscope for comparison with opacity observed in hatched chicks. The opaque areas for lenses of 9-day embryos were found to be approximately the same size regardless of dosage of DNP employed. Opacities in hatched chicks injected with 500 μg of DNP were approximately the same size and of the same nature as 9-day embryos. However, with smaller doses of DNP the size of cataracts in the hatched chick was smaller than that observed in 9-day embryos. This suggests that the DNP had no further cataractogenic action after the initial opacity was produced. This also suggests that some regression of opacity occurred with diminishing dosage of DNP.

DNP injections failed to produce a cataract when injected on fifth day. At this time the proximal wall of embryonic lens sac approaches the distal wall(10). The latter becomes the anterior marginal epithelium of the

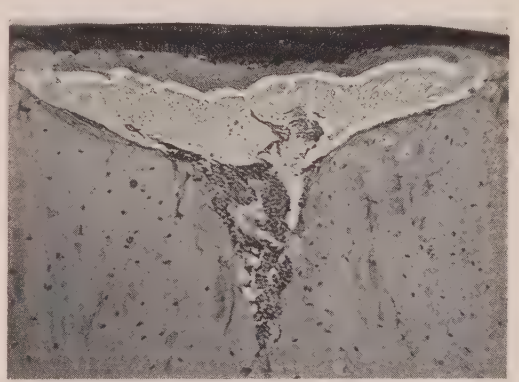

FIG. 1. Degenerative area in microscopic section through lens of hatched chick inj. with 500 μg of DNP on 8th day of incubation. (Hematoxylin and eosin) 58×.

lens. The cataract formed when DNP was injected on 8th day of incubation occurred when the 2 walls were apparently in contact (10). The oxidative enzyme systems are found only in epithelium, and the cortex is dependent on the epithelium for its metabolism(2,9). It is possible that this relationship may be involved in cataract formation.

Several hatched chicks were sacrificed for histological studies. Fig. 1 shows the lens of a newly hatched chick which received 500 μg of DNP on 8th day of incubation. There is a degeneration of lens fibers and a liquefaction of lens protein. This degeneration in the lens of the chick is similar to that previously observed in Vit. E-deficient turkey embryos(5,11).

Seventy-seven eggs were injected with 100 and 750 μg of DNP after 15 days incubation. The 100 μg level had no effect. The 750 μg level resulted in lens opacities in embryos from 20 hour post-injection sample and live-unhatched embryos. Hatched chicks appeared normal.

Summary. 1) Injections of 1-25 μg of DNP into chicken eggs, prior to incubation resulted in embryonic mortality after 3 days incubation, but did not produce lens changes. 2) Injections of 25-200 μg of DNP after 5 days incubation did not produce lenticular changes. 3) Cataracts were produced in embryos and hatched chicks from eggs injected with 200-500 μg of DNP after 8 days incubation. The size of cataract was roughly the same in hatched chicks as in embryos from the 500 μg group. 4) Injection of 750 μg of DNP after 15 days incubation produced an opacity evident after 20 hours. However, the opacity apparently regressed since the eyes of chicks hatched from this group appeared to be normal.

1. Horner, W. D., *Trans. Am. Ophth. Soc.,* 1941, v77, 505.

2. Robbins, B. H., *J. Pharm. Exp. Ther.,* 1944, v80, 264.

3. Bettman, J. W., *Am. J. Ophth.,* 1946, v29, 1388.

4. Buschke, W., *ibid.,* 1947, v30, 1356.

5. Ferguson, T. M., Atkinson, R. L., Couch, J. R., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 868.

6. Landauer, W., 1951, Bull. No. 262, Storrs Agric. Exp. Sta., Univ. of Conn., Storrs, Conn., 161.

7. Needham, J., *Biochemistry and Morphogenesis,* 1952, Univ. Press, Cambridge.

8. Kinsey, V. E., Frohman, C. E., *Arch. Ophth.,* 1951, v46, 536.

9. Frohman, C. E., Kinsey, V. E., *ibid.,* 1952, v48, 12.

10. Hamilton, H. L., *Lillie's Development of the Chick,* 3rd ed., 1952, Henry Holt, N. Y.

11. Ferguson, T. M., Rigdon, R. H., Couch, J. R., *Arch. Ophth.,* 1956, v55, 346.

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Effect of Chlorpromazine on Swimming Time of Rats at Different Temperatures. (24138)

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Recently an analysis was made of the factors responsible for the marked hypothermia observed in rats treated with chlorpromazine (1). Chlorpromazine has also been shown to induce some muscle paralysis in rats(2). We have therefore studied the effect of chlorpromazine on swimming time of rats in water bath at different temperatures.

Methods. Male albino rats weighing 200 g were used. Swimming time of control and chlorpromazine-treated groups, composed of 6 rats, was measured when animals were swimming at 9, 19, 28 or 32°C. Chlorpromazine† 10 mg/kg was injected intraperitoneally 5 minutes prior to beginning of swimming and the rectal temperature was recorded with thermocouples. Room temperature was maintained at 21°C and water bath remained at

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† Chlorpromazine was graciously supplied by Smith, Kline and French (under trade name Thorazine).

TABLE I. Effect of Bath Temperature on Swimming Time of Control and Chlorpromazine (CPZ) Treated Animals.

Bath temp., °C	Swimming time, min. Control	CPZ†
9	9 ± 1*	9 ± 1
19	29 ± 7	18 ± 1
28	>90	22 ± 4
32	>90	>90

* Stand. error. † CPZ dose was 10 mg/kg.

constant temperatures for duration of experiment. In all experiments control and experimental animals swam at same time.

Results. When animals swam more than 1½ hr, the experiment was terminated, for it was observed that extremely wide variation occurred among normal animals in the time necessary to attain the limit of endurance in swimming. Furthermore, after 1½ hr the sedative and hypothermic effects of chlorpromazine disappeared gradually. Control animals swam longer than 1½ hr at 28 or 32°C, whereas at 19 or 9°C the swimming time dropped to 29 and 9 minutes, respectively (Table I). Chlorpromazine-treated animals swam longer than 1½ hr when the bath temperature was 32°C, whereas at 28, 19 and 9°C the swimming time (Table I) was greatly reduced. At bath temperature of 19°C, the rectal temperature drops at the same rate in the control as it does in chlorpromazine-treated animals (Fig. 1). The rectal temperature of control animals was 21.7°C when drowning occurred after 29 minutes of swimming, whereas the corresponding values

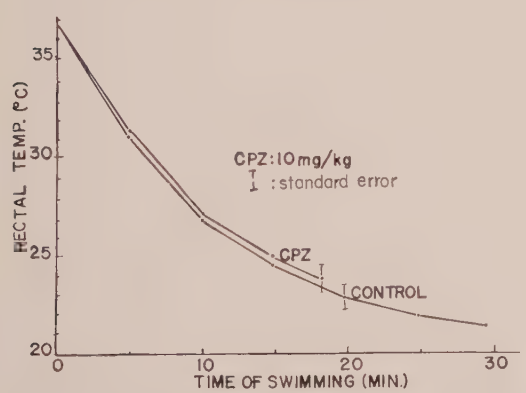

FIG. 1. Effect of swimming time on rectal temperature of control and chlorpromazine-treated animals swimming at 19°C.

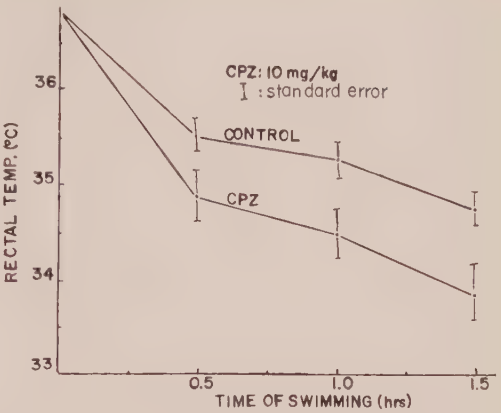

FIG. 2. Effect of swimming time on rectal temperature of control and chlorpromazine-treated animals swimming at 32°C.

for chlorpromazine-treated animals were 23.4°C and 18 minutes, respectively. When bath temperature is 32°C, the rectal temperature drops faster in chlorpromazine-treated group than in the control although both groups swim longer than 1½ hr (Fig. 2). When both groups swam the chlorpromazine-treated group was less disturbed and swam slower than the control group.

Discussion. Lowering of body temperature not only decreases speed of chemical reactions responsible for muscular contraction but also induces at certain levels a more or less pronounced state of narcosis. These responses to hyperthermia are probably the main factors responsible for decreased swimming time observed at certain bath temperatures in control animals. Because of muscle paralysis and sedation caused by chlorpromazine, it was suspected that this drug would potentiate the detrimental effect of low temperature on swimming animals. Sedation and muscular impairment in chlorpromazine-treated animals swimming at 32°C, probably explains the more rapid rate of body temperature drop. In spite of these effects the swimming time of both this group and the control exceeded 1.5 hr. However, at 28 and 19°C these reactions to chlorpromazine have a significant effect on swimming time. At 19°C the fall of body temperature was not faster in the experimental than in the control group, which indicates that factors other than changes in body temperature are responsible

for the difference in swimming time in both groups studied. It seems possible then that muscle paralysis and central sedation caused by both chlorpromazine and lower bath temperatures (28 or 19°C) become additive and explain the detrimental effect of chlorpromazine on animals swimming at temperatures lower than 32°C. At 9°C the effects of cold alone are so drastic and telescoped that chlorpromazine fails under these conditions to display its action.

Summary. When rats are swimming at 19 or 28°C, swimming time is markedly reduced by chlorpromazine (10 mg/kg) treatment. This difference is not due to differences in body temperature but is probably caused by sedation and muscular paralysis caused by this drug. At 9°C the detrimental effect of bath temperature on swimming time is so marked that chlorpromazine fails to display any action whereas at 32°C the stress is so small as to allow the animal to perform as well even when treated with chlorpromazine.

1. LeBlanc, J., *J. App. Physiol.*, 1958, v12.
2. Kopera, J., Armitage, A. K., *B. J. Pharmacol.*, 1954, v9, 392.

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Increase of Hexobarbital Sleeping Time by Certain Anticholinesterases.* (24139)

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Some organic phosphate insecticides, such as octamethyl pyrophosphoramide (OMPA) (1), p-nitrophenyl diethyl thionophosphate (parathion)(2) and ethyl-p-nitrophenyl thionobenzenephosphonate (EPN)(3) are converted by liver slices from relatively inactive to potent cholinesterase inhibitors. Conversion of OMPA(4), parathion(5), and hexobarbital(6) is an oxidative process, taking place in combined microsome and supernatant fractions of liver homogenates. Other organic phosphate anticholinesterases, such as S-(1,2-dicarbethoxyethyl)-O, O-dimethyl dithionophosphate (malathion), p-nitro-m-chlorophenyl dimethyl thionophosphate (chlorothion), and a mixture of bis-(dialkoxyphosphinothioyl) disulfides (phostex) are partially inactivated by liver slices(7). Since the alteration of these anticholinesterases by liver may involve the same enzyme systems as those detoxifying hexobarbital, their effects on duration of action of the latter were examined. Tetraethyl pyrophosphate (TEPP), and technical bis - dimethylamido - fluorophosphate (BFP) were used as examples of anticholinesterases not markedly activated nor rapidly inactivated by liver.

Methods and materials. Young adult male Swiss-Webster mice fasted 16 to 20 hours were used. Anticholinesterases† were given orally in corn oil 2 hrs before intraperitoneal injection of sodium hexobarbital in saline. TEPP was given to one group of mice 0.5 hr before the barbiturate. Sleeping time was the duration of loss of righting reflex from the side position.

Results. The highest doses of malathion and chlorothion and doses of other anticholinesterases used killed approximately 10% of the animals, all before injection of hexobarbital. Malathion, phostex and chlorothion produced the greatest prolongation of sleeping time (Table I). EPN and OMPA were also active, while BFP and TEPP were inactive.

Since malathion and chlorothion markedly prolonged sleeping time, it was expected that

* This investigation was supported in part by Office of Surgeon General, Department of Army.

† Chlorothion, OMPA and BFP were supplied by Dr. K. P. DuBois of University of Chicago; EPN by E. I. duPont de Nemours Co.; malathion by Cyanamid Co.; phostex by Niagara Chemical Division, Food Machinery and Chemical Corp.; TEPP by Victor Chemical Works.

TABLE I. Effect of Anticholinesterases on Duration of Hexobarbital (80 mg/kg) Sleeping Time.

Anticholinesterase, mg/kg	No. of mice	Sleeping time (min.), mean ± S.E.		P
	11	26.5	5.0	
EPN-30	11	85.3	13.3	<.01
	5	14.2	2.9	
OMPA-10	9	41.1	7.1	<.02
	15	32.4	5.9	
Malathion-500	17	116.1	15.3	<.01
	5	35.4	7.0	
-125	9	90.6	17.9	<.05
	6	18.0	7.4	
- 60	6	28.5	4.7	> "
	15	40.0	9.2	
Chlorothion-500	20	218.9	23.4	<.01
	5	39.6	10.7	
-125	10	115.9	10.3	< "
	6	27.8	9.3	
- 60	6	69.0	15.0	<.05
	5	34.4	6.2	
Phostex-1000	6	137.5	18.0	<.01
	5	16.6	5.6	
BFP-0.5	9	22.2	5.5	>.05
	15	23.3	3.2	
TEPP-2	11	32.6	7.3	> "
TEPP-2*	10	29.9	7.2	> "

* TEPP inj. .5 hr before hexobarbital.

they would also increase toxicity of hexobarbital. In groups of 10 animals each, however, 500 mg/kg of malathion or chlorothion did not significantly alter the toxicity of 200 or 250 mg/kg, approximately the LD_5 and LD_{50} of this barbiturate. Nearly all deaths occurred within 30 minutes after injection of the barbiturate, although at these doses animals surviving hexobarbital alone slept an average of 2 hours.

In agreement with our findings that anticholinesterases which are not rapidly altered by liver do not prolong hexobarbital sleeping time, it has been reported that physostigmine does not affect hexobarbital sleeping time(8). However, administration of physostigmine to mice before injection of barbital hastens the onset of action of this barbiturate and increases the rate of its entrance into the brain, an effect attributed to inhibition of cholinesterase(9). If chlorothion has a similar effect, this might partly explain its prolongation of the action of hexobarbital, although increased rate of penetration may not necessarily be accompanied by an increased duration of action. To determine whether chlorothion and TEPP alter the rate of penetration of sodium barbital into the brain their effects on the time from its intraperitoneal injection to loss of the righting reflex was measured (Table II). Since chlorothion did not hasten the onset of action of barbital, it is evident that it did not increase the rate of entrance of barbital into the brain. TEPP had an effect (Table II) similar to that of physostigmine(9), indicating that TEPP may also increase penetration of barbital into the brain. The negative results obtained with chlorothion suggest, however, that factors other than cholinesterase inhibition may be involved in the effect of anticholinesterases on brain permeability to barbital.

Discussion. Since hexobarbital is oxidatively inactivated by liver the possibility is suggested that the anticholinesterases which are rapidly activated or inactivated by liver prolonged hexobarbital sleeping time by competing for the enzyme system responsible for oxidation of the barbiturate. Though the enzymatic characteristics of the liver inactivation of malathion, chlorothion and phostex are unknown the finding that these agents prolonged hexobarbital sleeping time, points to the possibility that they compete for the enzyme system which detoxifies hexobarbital. It is significant that TEPP and technical BFP, which are not markedly affected by liver, did not prolong the action of hexobarbital.

Failure of malathion and chlorothion to affect toxicity of hexobarbital might be attributed to failure to increase, within the first half hour, the maximum concentration of the barbiturate in the central nervous system, in

TABLE II. Effect of Chlorothion and TEPP on Time of Onset of Action of Sodium Barbital (300 mg/kg).

Anticholinesterase, mg/kg	No. of mice	Anesthetic lag (min.), mean ± S.E.		P
	10	32.4	1.7	
Chlorothion-500*	14	27.9	2.8	>.05
	8	40.0	7.5	
TEPP-2†	12	25.3	1.5	< "

* Inj. 2 hr before barbital.
† " .5 hr " " .

spite of inhibition of its oxidation by the liver. Since nearly all deaths from hexobarbital alone or combined with these agents occurred within the first half hour, it also appears that malathion and chlorothion did not maintain a sufficiently high concentration of barbiturate over a long enough time to be lethal.

The effect of these anticholinesterases on concentration of hexobarbital in some tissues of these animals, and their effect on duration of action of other barbiturates and other drugs which are oxidatively metabolized by liver are being investigated. In a preliminary test pentobarbital sleeping time in mice was also markedly prolonged by chlorothion. Such findings may form the basis for caution by those whose work cause exposure to this type of insecticide and for whom barbiturates or other drugs oxidized by liver are simultaneously prescribed.

Summary. Organic phosphate insecticides, OMPA, EPN, malathion, chlorothion and phostex, which are rapidly metabolized in liver, markedly increased hexobarbital sleeping time in mice, while BFP and TEPP did not. Malathion and chlorothion did not alter toxicity of hexobarbital. TEPP hastened onset of action of barbital while chlorothion did not. Certain anticholinesterases which are rapidly metabolized by liver may compete for enzymes which are responsible for destruction of hexobarbital.

1. DuBois, K. P., Doull, J., Coon, J. M., *J. Pharm. and Exp. Ther.*, 1950, v99, 376.
2. Diggle, W. M., Gage, J. C., *Nature*, 1951, v168, 998.
3. Murphy, S. D., DuBois, K. P., *Fed. Proc.*, 1956, v15, 462.
4. Davison, A. N., *Biochem. J.*, 1955, v61, 203.
5. O'Brien, R. D., *Canad. J. Biochem. and Physiol.*, 1956, v34, 1131.
6. Cooper, J. R., Brodie, B. B., *J. Pharm. and Exp. Ther.*, 1955, v114, 409.
7. Rosenberg, P., Coon, J. M., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 836.
8. Beiler, J. M., Brendel, R., Martin, G. J., *J. Pharm. and Exp. Ther.*, 1956, v118, 415.
9. Greig, M. E., Mayberry, T. C., *ibid.*, 1951, v102, 1.

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Alterations in Rat Serum Proteins in Single Deficiencies of Folic Acid and Vit. B_{12}.* (24140)

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The influence of a deficiency of folic acid (PGA) and Vit. B_{12} on rat serum proteins was reported(1). A lowering of total protein level was attended by reductions in serum concentrations of albumin, α_1-globulin and γ-globulin. An increase in proportion of β-globulin was interpreted as indicative of higher serum level of free Vit. B_{12} in the deficient state. In continuation of this work, the effects due to single deficiencies of Vit. B_{12} and PGA on serum proteins and Vit. B_{12} have been evaluated and are here reported.

Materials and methods. Young male rats (Wistar strain), 50 g weight, were depleted of their Vit. B_{12} and PGA reserves by maintenance on a deficient iodo-casein diet containing succinyl sulphathiazole(1) for 4 weeks. The animals were then divided into 4 comparable groups and were fed the following diets: (a) basal ration, (b) basal ration plus Vit. B_{12}, (c) basal ration plus PGA and (d) basal ration plus Vit. B_{12} and PGA. The basal ration consisted of (g/100 g of diet): Vit.-free casein, 10; succinyl sulphathiazole, 2; arachis oil, 6; shark liver oil, 2; salt mixture (U.S.P. No. 2), 4; sucrose, 10; and maize starch, 66, with vitamin additions as stated previously(1). Additions of Vit. B_{12} and PGA, where made, were at levels of 50 μg and

* This work was supported by a grant from the Williams-Waterman Fund of Research Corp., N. Y. City.

TABLE I. Blood and Liver Picture in Deficiencies of Vit. B_{12} and Folic Acid. Results represent mean values for 6 rats ± S.D.

Supplement	Erythrocytes/mm³ (× million)	Hemoglobin, g/100 ml	Leucocytes/mm³ (× 1,000)	Liver PGA, μg/g	Liver vit. B_{12}, mμg/g
PGA + vit. B_{12}	6.2 ± .8	14.8 ± .4	7.1 ± 2.6	7.2 ± 2.1	92.8 ± 20.7
PGA	4.4 ± 1.1	12.3 ± .9	6.2 ± 1.4	5.4 ± 1.7	34.5 ± 15.9
Vit. B_{12}	5.6 ± .9	12.6 ± .7	4.9 ± .9	2.1 ± .3	78.4 ± 13.9
None	3.1 ± 1.4	10.8 ± 1.3	3.6 ± 1.2	2.4 ± 1.5	28.6 ± 17.9

5 mg respectively/kg diet. After a further period of 5 weeks the animals were dissected under anesthesia, and blood obtained from the inferior vena cava. A portion was immediately heparinized for cell count and hemoglobin determinations and the rest allowed to clot at 37°C for 1 hour and later centrifuged at 2°C to obtain the serum. Livers were quickly excised and chilled in cracked ice. Hemoglobin was determined by acid hematin method. Vit. B_{12} in serum was determined as total and free vitamin, by the method of Ross (2) using *Euglena gracilis* as test organism. Assays were in triplicate using 50 μl serum in 4 ml basal medium. Total Vit. B_{12} was determined after liberating bound vitamin by heating mixture at 100°C for 15 minutes. For determination of free vitamin, the mixture was sterilized at 56°C for 30 minutes prior to inoculation of test organism. Growth was measured turbidimetrically after 8 days using Klett-Summerson photo-colorimeter with 660 mμ filter in position. Total serum was determined by biuret method(3). Fractionation of serum proteins was carried out by electrophoresis on Whatman No. 3 paper strips as described previously(1). The separated proteins were stained with bromophenol blue by method of Jencks *et al.*(4). Relative percentages of the separated protein components were evaluated densitometrically. Liver PGA was determined after autolysis of tissue in 0.2 M phosphate buffer of pH 7.6 using *Streptococcus faecalis R* (ATCC 8043) as test organism and assay medium of Mitbander and Sreenivasan(5). Liver Vit. B_{12} was determined after liberation from tissue by overnight incubation under toluene with papain in 0.1 M acetate buffer of pH 4.6. Assays were made with *Lactobacillus leichmannii* (ATCC 7830) by a turbidimetric adaptation of the U.S.P. method(6).

Results. The results of blood and liver analyses for control and deficient groups of animals are summarized in Table I. Table II summarizes data on serum proteins and Vit. B_{12}. Typical electrophoretic profiles for control and deficient groups are reproduced in Fig. 1.

While manifestations of single and combined deficiencies are as may be expected, modifications in serum protein profile induced by Vit. B_{12} deficiency are different from those induced by PGA deficiency. In Vit. B_{12} deficiency, the decrease in total protein is characterized by a drop in albumin and in α_1-globulin fractions. In PGA deficiency, on the other hand, β- and γ-globulins are also affected. Reductions in albumin and in α_1-globulin fractions, however, are significantly greater in Vit. B_{12} deficiency than in PGA deficiency. Reductions in albumin, α_1-globulin

TABLE II. Serum Proteins and Vit. B_{12} in Folic Acid and Vit. B_{12} Deficiency. Results represent mean values for 6 rats ± S.D.

Supplements	Serum proteins						Serum vit. B_{12}	
	Total protein	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Total	Free
	g/100 ml serum						μμg/ml serum	
PGA + vit. B_{12}	5.65 ± .21	2.30 ± .05	1.05 ± .08	.46 ± .10	.86 ± .08	.97 ± .12	730.6 ±102.4	206.4 ± 98.2
PGA	4.89 ± .34	1.65 ± .11	.88 ± .07	.49 ± .05	.85 ± .05	1.02 ± .07	146.0 ± 39.8	85.2 ± 26.2
Vit. B_{12}	4.70 ± .21	1.93 ± .08	.98 ± .04	.47 ± .08	.63 ± .06	.68 ± .09	614.2 ± 78.8	190.0 ± 44.3
None	4.18 ± .38	1.48 ± .09	.74 ± .05	.52 ± .12	.90 ± .07	.54 ± .14	120.4 ± 32.0	69.8 ± 28.4

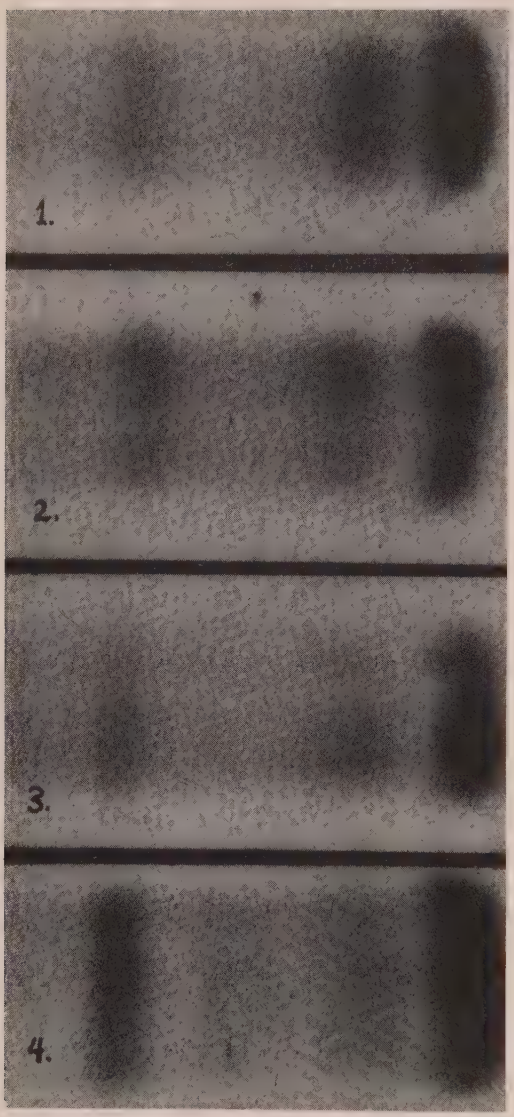

FIG. 1. Alteration in electrophoretic pattern of serum proteins in deficiencies of PGA and vit. B_{12}. Patterns 1-4 represent typical profiles, respectively, of control, vit. B_{12} deficient, PGA deficient, and vit. B_{12} and PGA deficient groups. The bands reading from the starting line are, in order, γ-globulin, β-globulin, α_2-globulin, α_1-globulin and albumin.

and γ-globulin in combined deficiency of the 2 vitamins exceed those in single deficiencies of either PGA or Vit. B_{12}. The relative concentration of β-globulin is markedly increased in Vit. B_{12} deficiency and in combined deficiency of Vit. B_{12} and PGA but not in single deficiency of PGA. There is a similar elevation in ratio of free Vit. B_{12} to total Vit. B_{12} in sera of animals deficient in Vit. B_{12} alone or in Vit. B_{12} and PGA. In PGA-deficient animals the ratio is essentially normal.

Discussion. Vit. B_{12} in human liver tissue exists in loose combination with a protein fraction resembling serum β-globulin in electrophoretic mobility(7). When added to human serum *in vitro,* it is found partly in combination with β-globulin fraction(7-9). In normal human sera, the α-globulin fraction is the primary source of the bound vitamin; free B_{12} found in 3 out of 13 samples corresponded in amount to that found in 3 β-globulin fractions(7). In our work(10) with the rat, serum α_2-globulin is chiefly responsible for binding of vitamin under normal conditions; however, a part may also remain associated with β-globulin when large amounts of the vitamin enter the circulation. Furthermore, a good parallelism has been obtained between quantity of vitamin found associated with α_2-globulin and bound vitamin in whole serum, and between free vitamin concentration and β-globulin-bound vitamin suggesting that, while α_2-globulin may be primarily responsible for immediate binding and storage of vitamin entering the circulation, β-globulin may aid in transporting the vitamin to its functional sites and depots in a manner analogous to iron transport mechanism of serum wherein a specific β_1-globulin is known to function by carrying iron to and from its deposits in tissues(11,12). The recent case report(13) that a patient with unexplained anemia had no β-globulin in circulation is of interest in this connection.

Ludovici and Axelrod(14) have demonstrated that ability of the rat to produce antibodies to human erythrocytes is markedly impaired in a deficiency of folic acid but not in Vit. B_{12} deficiency. The present findings, that serum γ-globulin concentration is decreased in PGA deficiency but is essentially normal in Vit. B_{12} deficiency, may suggest that Vit. B_{12} and PGA play similar roles in γ-globulin and antibody syntheses.

Summary. 1. In Vit. B_{12} deficient rat there is a decrease in serum levels of albumin and α_1-globulin. In PGA deficiency these fractions are affected to a lesser extent but

there is, in addition, a significant drop in levels of β- and γ-globulins. Reductions in serum levels of albumin, α_1-globulin and γ-globulin in combined deficiency of Vit. B_{12} and PGA are greater than those in single deficiencies of either vitamin. 2. In Vit. B_{12} deficiency, an increase in proportion of β-globulin is attended by increase in relative concentration of free Vit. B_{12} in serum. It is suggested that there may occur a preferential synthesis of β-globulin in the deficient state whereby mobilization of the available vitamin may be effected. 3. The observed alterations in serum level of γ-globulin are similar to the known effects of corresponding vitamin deficiencies on antibody synthesizing ability of the animal.

1. Mulgaonkar, A. G., Sreenivasan, A., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 44.

2. Ross, G. I. M., *J. Clin. Path.*, 1952, v5, 250.

3. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.

4. Jencks, W. P., Jetton, M. R., Durrum, E. L., *Biochem. J.*, 1955, v60, 205.

5. Mitbander, V. B., Sreenivasan, A., *Arch. f. Miokrobiol.*, 1955, v21, 60.

6. Rege, D. V., *Ph. D. Thesis, Univ. of Bombay*, 1953.

7. Pitney, W. R., Beard, M. F., VanLeon, E. J., *J. Biol. Chem.*, 1954, v207, 143; 1955, v212, 117.

8. Latner, A. L., Zaki, A. H., *Biochem. J.*, 1957, v66, 54.

9. Miller, O. N., *Arch. Biochem. Biophys.*, 1957, v72, 8.

10. Mulgaonkar, A. G., Sreenivasan, A., *Ind. J. Med. Res.*, 1958, in press.

11. Cartwright, G. E., Wintrobe, M. M., *J. Clin. Invest.*, 1949, v28, 86.

12. Surgenor, D. E., Koechlin, B. A., Strong, L. E., *ibid.*, 1949, v28, 73.

13. Riegel, C., Thomas, D., *New Engl. J. Med.*, 1956, v255, 434.

14. Ludovici, P. P., Axelrod, A. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 526.

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Some Systemic Chemical Responses to Local Inflammation.* (24141)

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Dunphy and Udupa(1) have recently described chemical alterations in rat skin during wound healing. Using simplified micromethods(2), we studied the effect of inflammation on hexosamine, nitrogen and hydroxyproline concentration of both necrotic and normal skin of rats injected intradermally with croton oil to produce localized injury.

Materials and methods. Wistar strain male rats weighing 200 to 300 g were used. Appropriate areas of shaved skin were removed from animals killed by etherization, dissected clean of adhering fat and muscle and stored in frozen state until analysis. The tissue was minced, and autoclaved 3 hours with 10 ml/g 4N HCl. Subsequent analytical procedures have been described(2). The results are expressed in terms of μmoles of hexosamine or hydroxyproline/mmole nitrogen. To provide controlled areas of necrosis, 0.4 ml croton oil (Magnus, Mabee and Reynard Co. N. Y.) was injected intradermally into a previously shaved area of rat skin. The preparation gave no gross evidence of wound healing for at least 7 days, but produced necrosis throughout an area of about one cm^2 within 3 days. The injury included dermis, epidermis and subcutaneous tissue with no suppuration and minimal sloughing. To demonstrate relative homogeneity of the chemistry of rat skin, 6 rats weighing 250 to 300 g were killed. The following 4 areas of whole rat skin were selected for study; ventral, mid dorsal, right foreleg and left hindleg. Ratio of hexosamine and hydroxyproline to nitrogen and of hexosamine to hydroxyproline for each area of skin was determined in each animal, and mean ratios and

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TABLE I. Mean Chemical Ratios of Various Skin Areas.

Ratio	Skin locations: Right foreleg	Left hindleg	Ventral	Dorsal
Hexosamine (μmole) / Nitrogen (mmole)	3.1 ± .9	2.7 ± .2	2.8 ± .7	2.8 ± .6
Hydroxyproline (μmole) / Nitrogen (mmole)	.26 ±52	119 ±18	107 ±19	124 ±28
Hexosamine / Hydroxyproline	.024 ± .005	.024 ± .004	.026 ± .005	.023 ± .003

TABLE II. Some Chemical Effects of Inflammation.

Days after injury	Hexosamine in: Injured skin	Hexosamine in: Control skin	Hydroxyproline in: Injured skin	Hydroxyproline in: Control skin	Hydroxyproline in: Blood
0	3.8 ± .3	3.8 ± .3	125 ± 13	125 ± 13	2.5 ± .5
3	*10.1 ± 1.7	4.7 ± 1.2	*75.3 ± 4.7	*90 ± 10.7	*8.9 ± .5
4	*14.3 ± 3.3	4.5 ± 1.2	*65.9 ± 53	*88.6 ± 12	*5.5 ± .2
7	*12.8 ± 3.6	*5.8 ± .9	*43.0 ± 8.0	109.0 ± 14	*6.1 ± .3

* Significantly different from 0 time (*i.e.*, $p < .02$).

standard deviations calculated.

Results. Table I indicates that there is no statistically significant variation in chemical analysis with skin locality ($p > .1$). Consequently, any area of skin would be representative of the whole tissue.

Nine male rats weighing 200 to 220 g were injected with croton oil. On 3, 4, and 7 days after injection 3 of these animals were killed. Five ml blood, the necrotic skin (primarily dermis with epithelial papillae), and an equivalent area of skin from the uninjured side were removed. Results of the analyses of hydroxyproline and nitrogen in serum and of hexosamine, hydroxyproline and nitrogen in corresponding skin areas are presented in Table II, along with normal values found in uninjured animals.

Hemolysis in blood samples contributed no appreciable color (tyrosine) to the determination of serum hydroxyproline concentration.

The results were analyzed for statistical significance by the "t" test, and significant changes from normal were found in 12 of the 15 determinations reported in Table II. The necrotic area showed a marked fall in hydroxyproline and a profound increase in hexosamine concentration, while the control area indicated smaller, but parallel, changes in these components. The hydroxyproline to nitrogen ratio of blood demonstrated a 4-fold increase during injury.

The hexosamine-hydroxyproline ratio of the necrotic tissue increased markedly with time after injury, while there was a similar, but considerably smaller change in this ratio for uninjured areas of skin (Fig. 1).

Discussion. One of the major difficulties in studying the chemistry of the inflammatory response is to provide a constant amount of necrotic tissue. At the suggestion of R. H. Pearce, we found that croton oil produced a very reproducible lesion. Intensity of inflammation at any time after injection may be

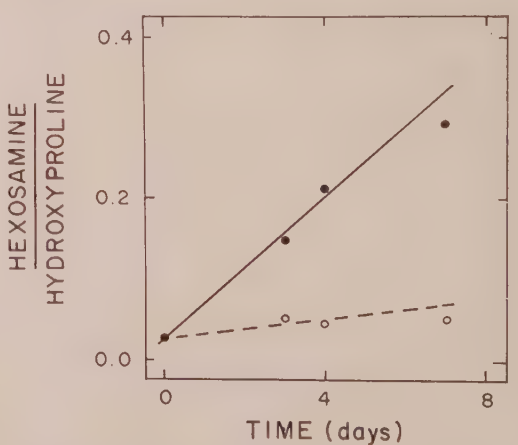

FIG. 1. Change in ratio of hexosamine to hydroxyproline in rat skin with time after injury. Injured tissue (●); uninjured tissue (○).

controlled by strength of irritant used. Pearce found that the effectiveness of various preparations of croton oil differed enormously (personal communication).

Of considerable importance to the study of inflammation is the assurance that variations in skin locality will not produce significant alterations in their chemical analyses. Table I provides this assurance. Consequently, the possibility that the area of skin opposite the site of injury differed in chemical content from area of injection is excluded.

Immediately after injection, the wounded animals lost weight, but after a few days this loss was essentially recovered. Corrections in skin analysis for weight loss or gain could be made from data recently published by us (2), although during the short time used in this study (7 days) alterations in body weight were not significant. This is not true for immature animals weighing 100-150 g (R. H. Pearce, unpublished). The increase in hexosamine and decrease in hydroxyproline or collagen measured during necrosis, was the exact inverse of that reported by Dunphy and Udupa(1) for wound healing. These workers studied preparations containing minimal necrotic tissue, while our animals displayed minimal healing. Presumably, superimposition of both groups of data would provide an overall picture of skin chemistry of inflammation and repair.

An increase in circulating concentration of hydroxyproline is logically consistent with rapid disappearance of this amino acid from skin. Although tyrosine from hemoglobin does not contribute appreciably to hydroxyproline analysis, serum proteins rich in tyrosine undoubtedly do. Concentrations of hydroxyproline in blood reported by Kivirrikko, Liesmaa and Lukainen(3) were considerably smaller than that measured by us. The difference is probably due to the effect of tyrosine upon our analysis. The magnitude of change in blood "hydroxyproline" with inflammation precludes the possibility that all changes in chromagenic material were due to alterations in serum protein tyrosine alone, particularly when the Martin and Axelrod procedure was used to determine hydroxyproline(4).

The striking, almost linear increase in hexosamine to hydroxyproline ratio with time after injury is paralleled by a small change in this ratio in uninjured tissue samples. This latter change is of borderline significance, despite the extremely significant change of each separate component. This is probably due to the fact that when the hexosamine change was significant, the hydroxyproline change was not, and *vice versa* (Table II).

Summary and conclusion. Chemical analysis of rat skin was independent of the area from which the sample was taken. Increases in hexosamine and decreases in hydroxyproline concentration found in skin necrotized with croton oil was paralleled to a lesser degree in uninjured skin. This systemic response was also found as an increase in circulating concentration of hydroxyproline in blood. Despite the statistical significance of these changes, the markedly increasing ratio of hexosamine to hydroxyproline in necrotic tissue with time was not paralleled significantly by the control.

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1. Dunphy, J. E., Udupa, K. N., *New Eng. J. Med.*, 1955, v253, 847.

2. Houck, J. C., Jacob, R. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 604.

3. Kivirrikko, K. I., Leismaa, M., Luukainen, T., *Acta Endocrinol.*, 1958, v27, 118.

4. Martin, C. J., Axelrod, H. E., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 461.

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Inhibition of Lipemia Clearing Activity by Tissue Extracts.* (24142)

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The serum of patients with primary or secondary hyperlipemia inhibits heparin-induced lipemia clearing activity(1). After high-speed centrifugation of hyperlipemic serum, inhibitory activity is located in the supernatant fatty layer(2). Seifter and Baeder(3) showed that a dialyzable inhibitor of lipemia clearing is present in the blood of rats. Hollett and Meng(4) separated a fraction from normal human serum which inhibited heparin-induced lipemia clearing activity. The studies presented here were undertaken in order to determine whether inhibitors of the lipemia clearing system are present in the tissues as well as in the blood. Such studies seemed to be further indicated by the presence of clearing activity in the tissues(5).

Materials and methods. Aliquots of a number of tissues were obtained from human autopsies† and from exsanguinated rabbits. *Tissue extracts* were prepared by differential centrifugation after repeated washing of tissue aliquots in 0.85% NaCl by a modification of the method of Schneider(6). Washed specimens were homogenized in one volume of saline at 5°C and centrifuged at 2500 g for 30 minutes at 4°C. The sediment was resuspended in one volume of saline and kept at −15°C for 24 hours.‡ After thawing the suspension was centrifuged as before. The supernatant was passed through a Seitz filter and further cleared of particulates by centrifugation at 32,000 g for 30 minutes at 4°C. (Saline Extract). *Solubility characteristics* of the inhibitory tissue components were determined in pH range between 2 and 12. In the acid pH range 0.1 M sodium citrate-citric acid buffers, and in the alkaline range 0.2 M sodium glycinate-glycine buffers were used. Extraction was carried out in the respective buffers as described above for saline. Extracts were dialyzed at 15°C against running tap water for 24 hours and distilled water for 1 hour, and then centrifuged at 32,000 g for 30 min. at 4°C. The supernatants (Glycine Extracts and Citrate Extracts, respectively) were kept at 5°C until used. *Effects of extracts* on heparin-induced clearing activity were determined by optical density measurements, glycerol evolution and electrophoretic mobility of lipoproteins. For these determinations 0.6 cc of extract was mixed with 0.6 cc of human post-heparin§ or fasting serum. To each mixture 0.1 cc of solution containing 100 N.I.H. units of thrombin‖/cc of physiological saline were added. Mixtures were incubated at 37°C for 10 min. and clots removed after fluid had been expressed from them. Then, 0.3 cc of standard fat emulsion¶ was added and the final mixture was incubated at 37°C. The degree of clearing was determined spectrophotometrically according to method of Grossman(7). Electrophoretic mobilities of serum alpha and beta lipoproteins in this mixture were determined by means of paper electrophoresis as previously described(8). Glycerol determinations were carried out on aliquots of mixtures prior to and after 1 hour incubation at 37°C with Korn's modification

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‡ Higher levels of inhibitory activity resulted from freezing and thawing the tissues.

§ Post-heparin serum prepared as previously reported(1).

‖ Thrombin (Topical Thrombin, Parke, Davis & Co., Detroit, Mich.) was added to remove residual fibrinogen which otherwise formed fibrin during optical density measurements and thus interfered with these determinations.

¶ The standard fat emulsion contained 15% coconut oil, 0.5% Pluronic (non-ionic detergent) and 1% polyglycerol oleate, supplied by Upjohn Co., Kalamazoo, Mich.

TABLE I. Inhibition of Heparin-Induced Lipemia Clearing Activity by Saline Extracts of Human Tissues.

Tissue	No. of specimens	Avg % of inhibition	Stand. dev.
Spleen	22	93	7
Kidney	24	90	7
Liver	21	89	8
Psoas muscle	7	78	8
Lung	6	68	11
Testis	5	62	19
Adrenals	8	47	12
Heart	14	38	20
Thyroid	3	30	8
Aorta	6	28	9
Adipose tissue	7	24	2
Skin	12	21	2
Brain	5	21	1
NaCl (control)		0	

(9) of the method of Lambert and Neish(10).

Results. Biochemical studies. Saline extracts of human and rabbit tissues inhibited lipemia clearing in post-heparin serum determined by optical density changes, glycerol evolution and electrophoretic mobilities of serum alpha and beta lipoproteins. There were differences, however, in inhibition by various tissues. Optical density determinations (Table I) revealed inhibition in extracts of all human tissues examined. Levels of inhibition in rabbit tissues corresponded with those in analogous human tissues. Variations in inhibition in any rabbit tissue were considerably less than in corresponding human tissue and did not exceed 8%. Liberation of glycerol from triglycerides in post-heparin serum was inhibited completely by saline extracts of tissues with strongly inhibitory activities (*i.e.*, kidney, spleen and liver). The method was not sufficiently sensitive for evaluating extracts which were moderately inhibitory by optical density measurements.

Increase in electrophoretic mobilities of alpha and beta lipoproteins, which reflects clearing activity in a mixture of fat emulsion with post-heparin serum, was inhibited by saline extracts of kidney, spleen and liver. Such inhibition did not occur, however, with saline extracts of other tissues. Sediments removed by high-speed centrifugation of kidney, spleen and liver extracts, when resuspended in saline, had between 10 and 30% of the inhibitory action of the first saline extracts. There was slight or no inhibitory activity in sediments obtained from other tissues.

Physical chemical studies. Dialysis of saline extracts of spleen, kidney, liver and heart against water or physiological saline resulted in 30 to 40% loss of inhibitory activity. Exposure of these extracts to increasing temperatures (15°-100°C) for 30 min. led to increasing loss of inhibitory activities. At 100°C the loss was 80 to 100%. Decreasing pH to 4.2 resulted in reversible precipitation of inhibitory components. Extraction with glycine buffer of human and rabbit kidney, spleen and liver was optimal at pH 7.5 to 8.0. In this pH range glycine extracts had between 80 and 100% of the inhibitory activities of corresponding saline extracts. Incubation of glycine extracts of kidney and liver with trypsin at 37°C for 12 hours resulted in 70 to 100% loss of inhibitory activities.

Control studies. Fasting serum was used in place of post-heparin serum in optical density determinations. No changes occurred, indicating that tissue extracts did not interfere with spectrophotometric determinations. Addition of heparin** either to tissues (kidney, spleen and liver) prior to extraction, or to extracts, did not effect their inhibitory activities. Use of Protamine in place of tissue extracts, in concentrations of 10μ/g/cc of saline did not inhibit clearing as determined by measurements of optical density and glycerol evolution.

Discussion. Our data indicate that extracts of a number of human and rabbit tissues inhibit heparin-induced clearing activity of serum, although degree of inhibition varied in different organs. Inhibition was least in tissues which normally have a high fat content (adipose tissue, brain) or which represent sites of pathological deposition of lipids (skin, aorta).

Loss of inhibitory activities of extracts which results from dialysis appears to be due to a dialyzable inhibitor. Since the inhibitory effects of the nondialyzable materials are

** Heparin sodium (Upjohn Co., Kalamazoo, Mich.) was added, 0.5 to 10 μg/g of tissue or /cc of tissue extract.

reduced by trypsin or heat, some of the inhibitory components appear to be proteins.

Failure of heparin to reduce inhibitory activities of tissue extracts furthermore suggests that inhibition is not the result of simple deactivation of clearing factor as a consequence of complex formation with polycations. Thus, *in vitro* inhibition of tissue clearing factor by Protamine which, as reported by Korn (8), is reversible by *in vitro* addition of heparin, differs from inhibition exerted by extracts described here.

Summary. Extracts of human rabbit tissues inhibited heparin-induced lipemia clearing activity of serum. Extracts of spleen, kidney and liver were strongly inhibitory; those of other organs exercised slight or moderate inhibition. The tissues contained inhibitory components which appear to be associated with proteins since they are nondialyzable, heat-labile and inactivated by trypsin.

1. Klein, E., Lever, W. F., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 565.
2. Lever, W. F., Klein, E., *J. Invest. Dermat.*, 1957, v29, 465.
3. Seifter, J., Baeder, D. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 42.
4. Hollett, C., Meng, H. C., *Fed. Proc.*, 1957, v16, 60.
5. Korn, E. D., *Science*, 1954, v120, 399.
6. Schneider, W. C., *J. Biol. Chem.*, 1946, v165, 585.
7. Grossman, M. I., *J. Lab. and Clin. Med.*, 1954, v43, 445.
8. Herbst, F. S. M., Lever, W. F., Hurley, N. A., *J. Invest. Dermat.*, 1955, v24, 507.
9. Korn, E. D., *J. Biol. Chem.*, 1955, v215, 15.
10. Lambert, M., Neish, A. C., *Can. J. Research*, 1950, v28, 83.

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Bioassay of Ataractics Against Lethal Action of Mescaline in Mice. (24143)

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Current interest in mescaline has stimulated a search for agents with antagonistic actions. Thus, Deegan and Cook(1) have reported the protective effects of various centrally active agents against pruritic episodes induced by mescaline in mice. Spech(2) reported that mescaline induced bradycardia and hypoglycemia in rats, which could be reduced with fasting and epinephrine. The same author reported that mortality was unaffected by the use of barbiturates, curare, decamethonium or physostigmine.

It is the purpose of this study to demonstrate the protective effects of various ataractic agents against mescaline-induced mortality in mice.

Methods. Toxicity of mescaline in female CF_1 mice (18-22 g) was determined using the intravenous route. The LD_{95} was found to be 150 mg/kg in 610 animals. The typical response consisted of a very brief clonic seizure terminated by respiratory arrest in a matter of 2 to 3 minutes. The agents under study were administered intraperitoneally one hour prior to treatment with mescaline, with the exception of the following: meprobamate (20 min), benactyzine (5 min), diphenylhydantoin (40 min), reserpine (24 hours), trimethadione (40 min), serotonin (30 min), morphine (30 min), and amphetamine (30 min). Only base weight of the drug was used in determining dosage. Ten mice were used per dose level and at least 3 doses per drug were examined. In this study the end point of measurement was the survival or death of the animal 24 hours following mescaline injection. The per cent protection was plotted on log-probit paper and the effective dose 50%, the slope, and the fiducial limits were calculated according to the methods of Litchfield and Wilcoxon(3).

Results. In Table I are shown the PD_{50}'s (protective dose 50%) and slopes of the protective agents with their respective fiducial

TABLE I. Protective Effects of Ataractics against Mortality Induced by Mescaline in Mice.

	PD_{50}	Fiducial limits	S	Fiducial limits
*Chlorpromazine	4.5	2.5-- 8.1	4.6	1.8–12
Perphenazine	4.5	3.1– 6.8	1.48	1.2– 2.0
Proclorperazine	10.0	7.3–13.5	1.7	1.4– 2.0
Thiopropazate	3.4	2.5– 4.6	1.5	1.2– 2.8
*Promazine	10.0	7.4–13.5	1.95	1.2– 3.1
Promethazine	15.0	13.1–17.1	1.3	1.1– 1.4
*Reserpine	2.5	1.4– 4.5	3.15	1.1– 8.8

* PD_{50} values only estimates since maximal protection exerted was 60 to 70%.

limits. As indicated, the most effective agents were thiopropazate, perphenazine, proclorperazine and promethazine with the following PD_{50}'s: 3.4, 4.5, 10.0 and 15.0 mg/kg. All 4 compounds brought about complete protection. On the other hand, reserpine, chlorpromazine and promazine did not exert complete protection and their PD_{50} values are only estimates since the maximal amount of protection was 60 to 70%.

No significant protection was obtained with the use of the following compounds at the dose ranges listed: sodium phenobarbital (3-96 mg/kg); meprobamate (10-160 mg/kg); benactyzine (3-40 mg/kg); atropine (1.5-48 mg/kg); hydroxyzine (Atarax)-(10-40 mg/kg); ethoxybutamoxane (3-96 mg/kg); SY-21 (N-ethyl-N-(9-fluorenyl)-2-aminoethylchloride HCl)-(1.5-16.0 mg/kg); diphenylhydantoin (5-20 mg/kg); trimethadione (1000-1500 mg/kg); LSD (Lysergic acid diethylamide) (1-4 mg/kg); serotonin (5-20 mg/kg); amphetamine (1-4 mg/kg); morphine (5-20 mg/kg); pyrilamine (Neo-antergan)-(3-48 mg/kg); diphenhydramine (Benadryl)-(6-24 mg/kg); iproniazid (Marsilid)-(10-800 mg/kg); pilocarpine (5-80 mg/kg); arecoline (5-80 mg/kg).

Discussion. The positive protective effects of chlorpromazine, perphenazine, proclorperazine, thiopropazate, promazine and reserpine against mescaline-induced mortality in mice have been demonstrated. The mechanism of action of these agents is probably central in nature for it is well known that they are characterized by a unique type of sedation. However, it has been reported that they also exert adrenergic blocking, anticholinergic, and antihistaminic properties(4,5). It was considered of interest to explore the possibility that one of these peripheral effects was active in protecting the animals. Agents classified as antihistaminics, anticholinergics, and adrenergic blocking agents, were examined for activity. Thus, peripheral adrenergic blockade produced by the specific adrenergic blocking agent, (N-ethyl-N-(9-fluorenyl)-2-aminoethylchloride hydrochloride)(6) and ethoxybutamoxane(7) did not prevent the toxic effects of mescaline. The anticholinergics, atropine and benactyzine, and the antihistaminics, pyrilamine and diphenhydramine, did not inhibit mortality. Finally, the sedatives and anticonvulsant, sodium phenobarbital, meprobamate and diphenylhydantoin, were found to exert no protection. In addition, stimulants and analgesics, represented in this study by amphetamine and morphine, were ineffective.

Since the antihistaminic, adrenergic blocking and anticholinergic agents were ineffective against the lethal effects of mescaline, it is highly probable that the protective effects of the ataractics are exerted centrally. Thus, Marrazzi and Hart(8,9) have reported that chlorpromazine and reserpine block the effects of mescaline in a 2-neuron intercortical (trans-

TABLE II. Inactive Agents against Mortality Induced by Mescaline in Mice.

Agent	Pretreatment time	Doses tested, mg/kg, I.P.
Sodium phenobarbital	1 hr	3, 6, 12, 24, 48, 72, 96
Meprobamate	20 min.	10, 20, 40, 80, 160
Benactyzine	5 "	3, 6, 10, 12, 20, 40
Atropine	1 hr	1.5, 3, 6, 12, 24, 48, 100
Hydroxyzine	1 "	10, 20, 40
Ethoxybutamoxane	1 "	3, 6, 12, 24, 48, 96
SY-21	1 "	1, 2, 4, 8, 16
Diphenylhydantoin	40 min.	5, 10, 20
Trimethadione	40 "	1000, 1250, 1500
Lysergic acid diethylamide	1 hr	1, 2, 4
Serotonin	30 min.	5, 10, 20
Amphetamine	30 "	1, 2, 4
Morphine	30 "	5, 10, 20
Pyrilamine	1 hr	3, 6, 12, 24, 48
Diphenhydramine	1 "	6, 12, 24
Iproniazid	1 "	10, 20, 40, 50, 100, 200, 400, 800
Pilocarpine	1 "	5, 10, 20, 40, 80
Arecoline	1 "	*Idem*

callosal) system. It would be, indeed, an interesting problem in mechanism of action to study the interactions of these agents at the enzymatic level.

Summary. 1. It has been demonstrated that chlorpromazine, perphenazine, proclorperazine, promazine, thiopropazate, promethazine and reserpine are effective antagonists of mescaline-induced mortality in CF_1 mice. 2. No significant protection was found with use of sodium phenobarbital, meprobamate, benactyzine, atropine, hydroxyzine, ethoxybutamoxane, SY-21, diphenylhydantoin, trimethadione, lysergic acid diethylamide, serotonin, amphetamine, morphine, pyrilamine, diphenhydramine, iproniazid, pilocarpine, and arecoline. 3. It is suggested that the protective effects of the ataractics against mescaline-induced mortality are centrally mediated.

1. Deegan, J. F., Cook, L., *J. Pharmacol. and Exp. Therap.,* 1958, v122, 17A.
2. Spech, L. B., *idem,* 1957, v119, 78.
3. Litchfield, J. T., Jr., Wilcoxon, F., *idem,* 1949, v96, 99.
4. Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., Koetschet, P., *Arch. int. pharmacodyn.,* 1953, v92, 305.
5. Bein, H. H., *Experientia,* 1953, v9, 107.
6. Stone, C. A., Komrad, E. L., Loew, E. R., *Arch. int. pharmacodyn.,* 1952, v92, 7.
7. Slater, J. H., Jones, G. T., *J. Pharmacol. Exp. Therap.,* 1958, v122, 69A.
8. Marrazzi, A. S., *Ann. N. Y. Acad. Sci.,* 1957, v66, 496.
9. Marrazzi, A. S., Hart, E. R., *Science,* 1955, v121, 365.

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Submicroscopic Morphology of Avian Neoplasms I. Studies on Erythroblastosis.* (24144)

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Electron microscope studies of ultrathin sections of cells infected with a number of different viruses have revealed the structure of various submicroscopic cell elements during different stages of infection and have showed the complicated structure of the viruses in cells and tissues. The observation that viruses can be detected in diseased tissues and that they can be differentiated from normal cell constituents has in turn led to electron microscope studies of tumors of known or suspected viral origin(1,2). The neoplastic conditions now classified in the chicken leukosis complex were the first tumors induced by filterable agents(3). Some tumors of the fowl leukosis complex, such as visceral lymphomatosis(4), osteopetrosis(4), erythroblastosis and myeloblastosis (granuloblastosis)(5) have been transmitted by cell-free preparations. Other conditions, such as neuro-lymphomatosis and ocular lymphomatosis, have not as yet been transmitted by cell-free preparations. Extensive studies have been carried out to ascertain the physical, chemical, immunologic and other properties of the viral agents present in cell-free preparations(5). Electron microscope studies on the morphology of particulate material in cell-free preparations, which had been dehydrated and shadowed, were also carried out to characterize agents responsible for erythroblastosis, myeloblastosis and lymphomatosis(5). Recently, the technic of ultrathin sectioning has been applied to ultracentrifugal pellets from plasmas of chickens with myeloblastosis and erythroblastosis(6). These studies revealed the characteristic structure of particles present in the ultracentrifugal

* Presented at March 1958 meeting of Am. Assn. for Cancer Research, Philadelphia. This work supported by Research Grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S., and Am. Cancer Soc.

pellets. Similar particles were also observed in ultrathin sections of spleen and bone marrow of chickens with erythromyeloblastosis (7). The present studies were undertaken to characterize the particles found in tissues of chickens with different forms of chicken leukoses. As a first step in ascertaining the etiologic relationship of the agents to the various chicken neoplasms, studies have been made of ultrathin sections of organs from chickens with erythroblastosis.

Materials and methods. The erythroblastosis strain was started by Engelbreth-Holm (8), and used extensively by Beard(5). Fourteen-day-old chickens of line 15, of U.S. Regional Poultry Research Laboratory, East Lansing, Mich., inoculated intravenously with a dose of log-4.0 ml of plasma from chickens with erythroblastosis, served as donors. Spleen and liver from chickens showing macroscopic and microscopic changes diagnostic of erythroblastosis were used for ultrathin section studies. Most material required, when in 70% alcohol, transportation to laboratory for 48 to 72 hours, after fixation for 1 hour in 1% osmic acid buffered with veronal acetate at pH 7.4. Storage of material in 70% alcohol had no deleterious effect. All steps, from immersion of tissue in a mixture of equal volumes of 100% alcohol and methacrylates (6 parts of n-butyl to 1 part of methyl) through final stage of polymerization at 48°C, following addition of Luperco to the methacrylates, were carried out in vacuum obtained by 15 lb of negative pressure. Sections were obtained with Porter-Blum microtome, and collected in 30% acetone on specimen screens covered with a thin film of collodion and thinly coated with evaporated carbon particles. An RCA EMU 3A electron microscope was used.

Results. Tumorous cells† in spleen and liver from chickens with erythroblastosis showed vacuolization of cytoplasm and alterations in mitochondria, endoplasmic reticulum, and cellular membranes. It should be pointed out that not all tumorous cells showed these changes. Frequently these changes were accompanied by appearance of osmiophilic bodies, often surrounded by single or double membranes. Within these inclusion-like bodies the spheroidal particles showing internal structure have been observed. Approximately one out of 50 cells examined showed the presence of these particles. Following breakdown of membrane surrounding the inclusion-like bodies, the characteristic particles are released into the surrounding cytoplasm. The nucleus of the affected cells remained unchanged. Characteristic particles have also been observed in intercellular spaces.

The particles have been found to vary in diameter from 570 Å to 760 Å, averaging 670 Å. These particles may be seen in Fig. 1, and at higher magnification in Fig. 1a. Their relationship to other cytoplasmic constituents is shown in Fig. 1. The ultrastructure is more clearly visible in Fig. 2 and Fig.

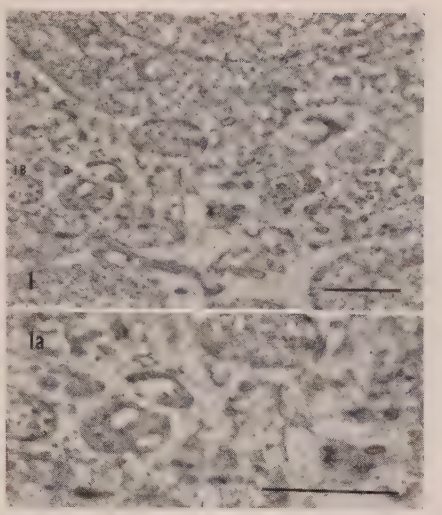

FIG. 1. Electron micrograph of section of tumorous cells of spleen from chicken with erythroblastosis. Inclusion-like bodies (IB) present in cytoplasm of some of the cells. Magnification 10,500 ×.

FIG. 1a. Part of Fig. 1 marked by "a" at greater enlargement. Two inclusion-like bodies with characteristic particles. Magnification 17,500 ×.

† Examination by means of the light microscope of spleen tissues from chickens in terminal stages of erythroblastosis reveals that the majority of all cells are primitive cells of erythroid origin. In ultrathin sections of erythroblastosis examined by means of electron microscope, it is difficult to differentiate the cells which show the described changes from those showing similar changes in granuloblastosis, which will be discussed later.

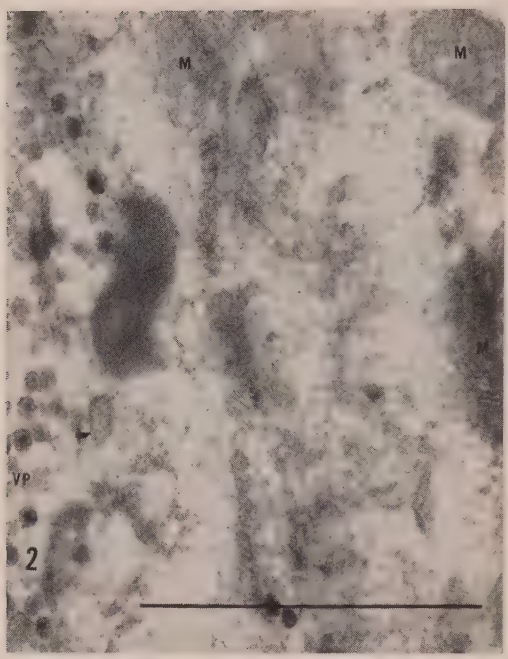

FIG. 2. Characteristic spheroid particles at greater enlargement showing internal structure; mitochondria (M). Magnification 46,667 ×.

3, 3a and 3b. The particles show a dense central mass, surrounded by a lighter zone, which in turn is surrounded by an external limiting membrane. Diameter of the central core was found to vary from 220 Å to 300 Å, averaging 260 Å.

Distribution of the particles both within and outside the cells is shown in Fig. 4, 4a, 4b, and 4c. The external limiting membrane is seen in particles present in inclusion-like bodies within the cytoplasm of cells and in particles found in extra-cellular spaces.

Discussion. The study of ultrathin sections of tumorous spleens and livers from chickens with erythroblastosis revealed a number of changes in the submicroscopic cell elements of some tumor cells. Some changes, like vacuolization of cytoplasm, breakdown of mitochondria, and of endoplasmic reticulum are not considered specific for viral infection. Similar changes have been observed in HeLa cells grown *in vitro*(8) and in cells subjected to starvation(9). However, other changes, like formation of inclusion-like bodies with spheroidal particles resembling virus particles observed in thin sections of other tumors of known viral origin(1) appear to be more specific.

Particles of similar internal structure but of somewhat larger diameter have been observed in plasma of chickens with erythroblastosis(6). The question arises about the relationship of the particles observed in ultrathin sections of tumorous spleens of chickens with erythroblastosis and those found in plasmas of chickens with erythroblastosis. The difference in size between particles in ultrathin sections of spleen and liver of chickens with erythroblastosis (670 Å) and those in ultrathin sections of ultracentrifugal pellets of plasma of chickens with erythroblastosis (800 Å) may be due to differences in preparation of the material and in calibration of the microscope. Spheroidal particles of similar ultrastructure and average diameter varying 750 Å to 800 Å were observed in ultrathin sections of spleen and bone marrow of

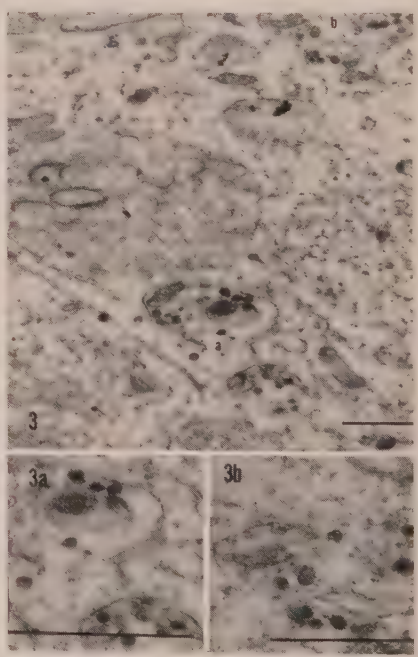

FIG. 3. Ultrathin section of several tumor cells in spleen of chicken with erythroblastosis. Characteristic "whorl-like" formation of membranous structures in cytoplasm of 2 cells. Inclusion-like bodies (a, b) with characteristic particles. Magnification 30,000 ×.

FIG. 3a and 3b. Parts of Fig. 3 enlarged. Characteristic structure of particles and membranes, in part broken-down, surrounding inclusion-like bodies. Magnifications 25,000 ×.

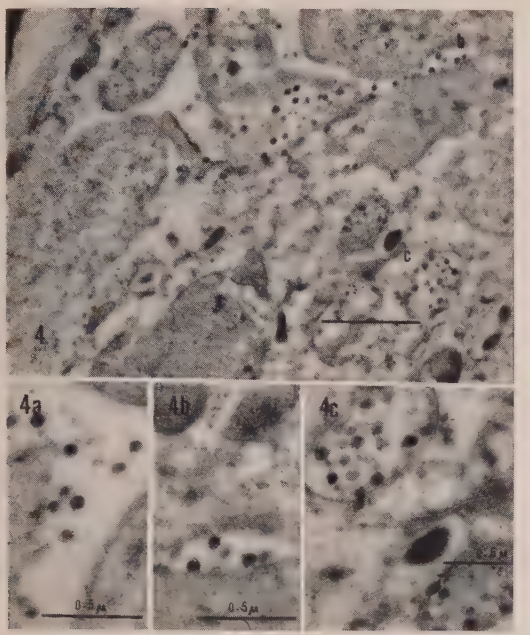

FIG. 4. Characteristic particles present both within and outside cytoplasm of cells of a tumorous spleen from a chicken with erythroblastosis. Magnification 25,000 ×.

FIG. 4a and 4b. Same particles in intercellular spaces. Magnification 25,000 ×.

FIG. 4c. Particles within inclusion-like bodies. Magnification 25,000 ×.

chickens with erythromyeloblastosis(7). Similar particles were also found in spleen and bone marrow of 3 out of 24 normal chickens (7). The size of these particles (750 Å-800 Å) resembles more closely the size of those (570 Å-760 Å) observed in the present study of thin sections of cells in erythroblastosis. It is possible that particles observed by Bernhard *et al.*(6) and by Benedetti *et al.*(7) and those in our studies represent the same agent. Inclusion-like bodies were also described in erythromyeloblastosis and their origin attributed to mitochondria(7). Similar inclusion-like bodies have been observed in the present study. Their origin will be discussed later.

The study of ultrathin sections of spleen and liver of control, young chickens of the same line, failed to reveal similar changes in the cells of these organs.

Summary. Ultrathin sections of tumorous spleen and liver from chickens of the so-called line 15 with erythroblastosis have been examined in the electron microscope. In cells of these organs spherical or spheroid structures were observed, composed of a central dense core of about 260 A, surrounded by a less dense material and limited by an outer membrane, varying in diameter from 570 Å to 760 Å. The structure of these particles resembles that of particles described in other tumors of chickens and in other tumors of known viral origin. No particles of similar character were observed in cells of organs from control healthy chickens.

1. Dmochowski, L., *Cancer,* v1, 216, Butterworth & Co., (Publishers) Ltd., London, 1957.

2. Swerdlow, M., Dalton, A. M., Birks, L. S., *Anal. Chem.,* 1956, v28, 597.

3. Ellermann, V., Bang, O., *Zentr. Bakteriol. Parasitenk.,* 1908, v46, 595.

4. Burmester, B. R., *Ann. N. Y. Acad. Sci.,* 1952, v54, 992.

5. Beard, J. W., *ibid.,* 1957, v68, 473.

6. Bernhard, W., Bonar, R. A., Beard, D., Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 48.

7. Benedetti, L. E., Bernhard, W., Oberling, Ch., *C. R. Acad. Sci.,* 1956, v242, 2801.

8. Morgan, C., Rose, H. M., Moore, D. H., *Ann. N. Y. Acad. Sci.,* 1957, v68, 302.

9. Gansler, H., Rouiller, C., *Schweiz. Z. Allgem. Path. u. Bakt.,* 1956, v19, 217.

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Submicroscopic Morphology of Avian Neoplasms. II. Studies on Granuloblastosis (Myeloblastosis).* (24145)

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Much valuable information about size and structure of a number of viruses has been gained by study of ultrathin sections of virus-infected cells. Examination of viruses in ultrathin sections of infected cells constitutes a convenient first step in investigation of ordinary and also of tumor-inducing viruses. This method provides a new approach to the study of viruses, their structure, mode of development, the part they play in the life of the cell, and also for similar investigations of tumors of suspected or unknown viral origin. Information gained in examination of ultrathin sections of tumors of known viral origin will undoubtedly be of help in the study of ultrathin sections of tumors of unknown viral etiology. The present studies were a continuation of investigations on viral agents involved in the origin of different forms of chicken leukosis complex, known to be transmitted by cell-free preparations. These studies constitute a first step in the attempts at characterization of different viral agents of chicken leukosis and assessment of their etiologic relationship in different types of chicken leukoses. A study of ultrathin sections of the affected organs of chickens with erythroblastosis has been reported(1). The present studies were undertaken to obtain information about changes in cells of the affected organs of chickens with granuloblastosis (myeloblastosis).

Materials and methods. The granuloblastosis strain was originated by Hall, Bean and Pollard(2) and studied extensively by Beard and his collaborators(3). Fourteen-day-old chickens of line 15, of the U. S. Regional Poultry Research Laboratory, East Lansing, Mich., were inoculated intravenously with a dose of log-1 ml of plasma from chickens with granuloblastosis. Chickens were killed on 19th day after inoculation and spleen and liver from chickens showing macroscopic and microscopic changes characteristic of granuloblastosis were used for ultrathin section studies. The method of tissue preparation for study in ultrathin sections was the same as for erythroblastosis(1). An RCA EMU 3 A electron microscope was used for study of the sectioned material.

Results. Tumorous† cells of spleen and liver from chickens with granuloblastosis (myeloblastosis) were found in ultrathin sections to have similar changes to those described in cells of the involved spleen and liver of chickens with erythroblastosis(1). In addition to changes in endoplasmic reticulum, mitochondria and the cell membrane, inclusion-like bodies were observed. These appear to be more frequently found in cytoplasm of tumorous cells (in approximately 1 out of 30 cells examined) than similar bodies in cells of erythroblastosis tumors. Characteristic particles with internal structure similar to that of particles in erythroblastosis have been found in inclusion-like bodies, freely scattered in cytoplasm of cells and also in intercellular spaces. The nucleus of the involved cells remained apparently unchanged. The outer diameter of the characteristic particles, showing an internal dense core, varied

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† After this manuscript was completed, a report on ultrathin sections of myeloblasts appeared (D. F. Parsons, Painter, J. C., Beaudreau, G. S., Becker, C., Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1958, v97, 839) in which it was indicated that virus particles were only rarely seen in myeloblasts but were numerous in other tissue cells. This is at variance with findings of this study, in which cells with described changes may be regarded as granuloblasts.

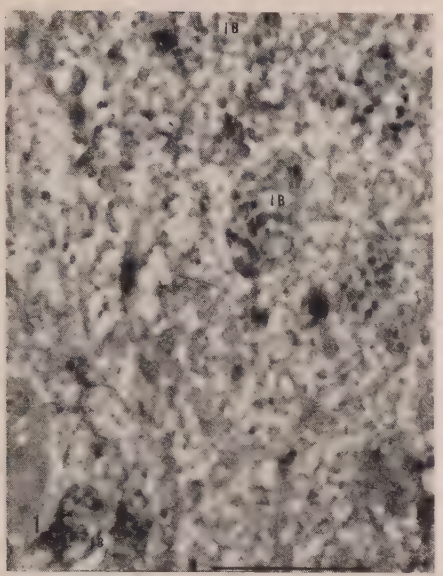

FIG. 1. Electron micrograph of ultrathin section of tumorous cells of spleen from chicken with granuloblastosis. Inclusion-like bodies (IB) with characteristic particles present in cytoplasm. Magnification 20,000 ×.

from 570 Å to 770 Å, with an average of 670 Å. Size of the dense center of the particles varied from 220 Å to 320 Å, averaging 260 Å.

An electron micrograph of an ultrathin section of several tumorous cells in spleen of a chicken with granuloblastosis is shown in Fig. 1. The characteristic feature of the picture is the considerable number of inclusion-like bodies frequently encountered in cytoplasm of the cells. At times, this cytoplasm was found almost entirely filled with these bodies containing the characteristic particles. Occasional particles lying free in the cytoplasm or within what may be extended endoplasmic reticulum may be seen in Fig. 1. Size of the inclusion-like bodies and number of particles within these bodies vary considerably. Most of the bodies are surrounded by a membrane, occasionally by a double membrane. Similar bodies at higher magnification are shown in Fig. 2. Some of the bodies show the characteristic particles in indistinct outlines. Some of the disorganized endoplasmic reticulum can also be seen. Inclusion-like bodies of somewhat different appearance are shown in Fig. 3 and 3a. These bodies, still surrounded by a distinct membrane, appear to be vacuoles in the cytoplasm containing large numbers of the characteristic particles. Some mitochondria showing changes are also seen. Altered mitochondria, and size which the inclusion-like bodies may reach are shown in Fig. 4. Particles at higher magnification present within the inclusion-like body are shown in Fig. 4a, an enlargement of Fig. 4. The ultrastructure of some of the particles may be clearly seen; also the dense material forming the central core of the particles may be seen either alone or partly surrounded by a limiting membrane. Some membranes without the dense center are also present. The same particles as they appear in intercellular spaces are shown in Fig. 5. There appears to be no difference in the ultra-structure between these particles and those within the inclusion-like bodies. However, a greater variation in size was encountered in particles lying outside the cells than in those present within the inclusion-like bodies.

Neither with respect to size nor to internal structure was there a distinction between particles found in ultrathin sections of the affected organs from chickens with erythroblastosis and granuloblastosis.

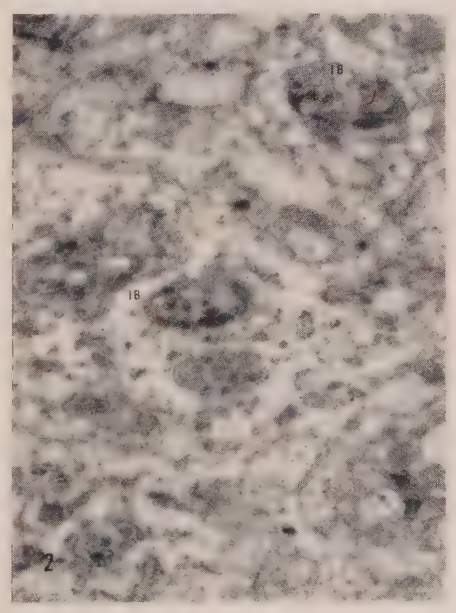

FIG. 2. Inclusion-like bodies (IB) in cytoplasm of tumorous cell with particles showing central core and peripheral membranes. Magnification 30,000×.

Discussion. Measurements of the size of characteristic spherical or spheroid particles observed in erythroblastosis and granuloblastosis gave almost identical values for both types of particles. Similar characteristic particles were observed in ultracentrifugal pellets from plasma of chickens with erythroblastosis and myeloblastosis (granuloblastosis) by Bernhard *et al.*(4) and in erythromyeloblastosis(6). It is possible that particles observed in our study and those found in myeloblastosis(4) represent the same agent. Measurements of the particle size in ultrathin sections of tumorous cells in erythroblastosis (1) and granuloblastosis differ somewhat from those reported by Bernhard *et al.*(4). Possible reasons for the difference were given previously(1).

FIG. 3. Inclusion-like bodies (IB) containing characteristic particles; mitochondria (M). Magnification 15,000 ×.

FIG. 3a. Part of Fig. 3 marked "a" at greater enlargement. Ultra-structure of particles is seen and membrane surrounding the vacuole. Magnification 35,000 ×.

The similarity in size and structure of particles observed in these 2 forms of the chicken leukoses need not necessarily indicate that they are the same agent. Chemical differences have been shown to exist between agents of erythroblastosis and myeloblastosis (granulo-

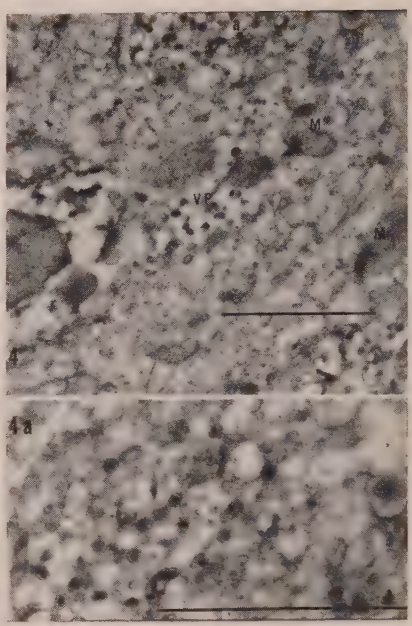

FIG. 4. Appearance of cytoplasm of tumorous cells from spleen of chicken with granuloblastosis. Mitochondria (M) showing changes. Characteristic particles (VP) scattered throughout cytoplasm and also present in inclusion-like body "a." Magnification 20,000 ×.

FIG. 4a. Part of Fig. 4, showing ultrastructure of characteristic particles. Magnification 35,000 ×.

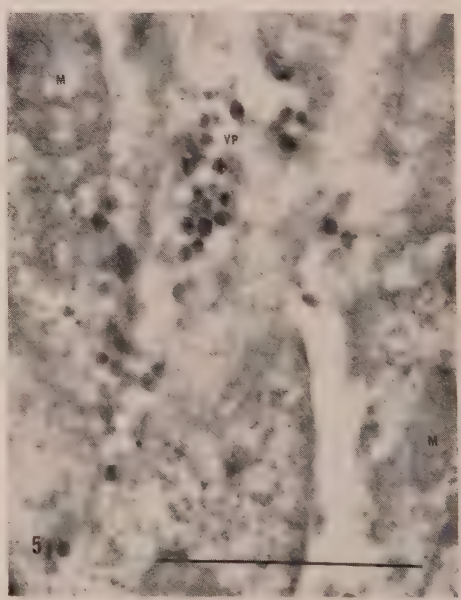

FIG. 5. Electron micrograph showing particles with similar ultra-structure present outside tumorous cell; mitochondria (M). Magnification 35,000 ×.

blastosis) by Beard and his collaborators(3). Whether the close immunologic relationship between the different agents of the leukosis complex(3) is indicatory of a close relationship between these agents and those of chicken tumors can only be settled finally when sufficiently pure preparations of the respective agents become available.

It does not appear probable that all agents involved in the origin of several chicken leukoses are identical, at least, in size. Electron microscope studies of ultrathin sections of spleen and liver of chickens with visceral lymphomatosis have been reported(5). The particles observed in the cells of the affected spleen were found to vary from 740 Å to 940 Å in diameter, with an average diameter of 840 Å. The size of their dense core ranged from 280 Å to 350 Å, with an average of 320 Å. These particles appear, therefore, to be considerably larger than those observed in erythroblastosis(1) and in granuloblastosis (myeloblastosis). It should be pointed out that the method used for preparing the material of visceral lymphomatosis for electron microscope studies was the same as that used for ultrathin section studies of erythroblastosis and granuloblastosis.

Summary. Tumorous spleens and livers from chickens with granuloblastosis were examined in ultrathin sections. In the cytoplasm of the affected cells changes were observed similar to those seen in erythroblastosis, another member of the fowl leukosis complex. Particles were observed in the cytoplasm of cells of similar size and structure to that of particles in erythroblastosis. Average total diameter of the particles was found to be 670 Å and that of the central dense core was 260 Å. No particles of similar size or structure were observed in ultrathin sections of organs from young healthy chickens of similar breed.

1. Dmochowski, L., Grey, C. E., Burmester, B. R., Fontes, A. K., PROC. SOC. EXP. BIOL. AND MED., 1958, in press.

2. Hall, J. W., Bean, C. W., Pollard, M., *Am. J. Vet. Research,* 1941, v2, 272.

3. Beard, J. W., *Ann. N. Y. Acad. Sci.,* 1957, v68, 473.

4. Bernhard, W., Bonar, R. A., Beard, D., Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 48.

5. Dmochowski, L., Grey, C. E., Burmester, B. R., *Proc. Am. Assn. Cancer Research,* 1957, v2, 196 (Abst.).

6. Benedetti, L. E., Bernhard, W., Oberling, Ch., *C. R. Acad. Sci.,* 1956, v242, 2801.

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Molt of Capon Feathering with Prolactin.* (24146)

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Our previously reported findings have shown that Progesterone (Pg) will initiate proliferation of the feather papilla with consequent molt in both sexes of fowl(1). Although the treatment interrupted ovulation for a period in the laying hen, the response of the male nonetheless made it seem possible that this activation of the papilla could be a direct effect of progestin. We attempted, unsuccessfully, to demonstrate a local effect of Pg upon the feather papilla(2); hence we made another approach to the question. Laying hens were treated with Pg and with other compounds of anticipated progestational activity while concurrently run experiments tested the effects of follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin (LTH) when administered singly and in conjunction with a Pg dose of established potency in molt(3). The various treatments (cortisone acetate excepted) all caused molt and also interrupted lay. There were, however, 2 important developments. With

* Scientific Paper A694. Contribution No. 2916 of the Maryland Agri. Exp. Station (Dept. of Poultry Husbandry).

testosterone propionate (TP), the lag between cessation of lay and initiation of molt was of such magnitude that a transformation of a portion of the injected TP to a progestationally effective compound, immediately active in the papilla, seemed plausible. The other and more interesting observation related to the effect of prolactin. The LTH treated hens showed by far the most rapid onset of molt with the least effect upon laying behavior. Furthermore, Pg did not augment the effects of LTH as it had those of FSH and LH when given jointly. Since it was nevertheless impossible to disentangle entirely the role of the ovary, it was decided to duplicate the more significant experimental conditions from this series in the male castrate: these findings are discussed here.

Methods. Capons were treated with Pg; FSH, FSH + Pg; LH, LH + Pg; LTH, LTH + Pg for the same period of time as had been employed in the hen. Pg was given as single dose of a compound having slow absorption from local depots.† The FSH, LH and LTH used concurrently alone and in combination with Pg were Armour's preparations.‡ A single LTH treatment, only, was later repeated with a different preparation§ in unused birds. Further details are entered in legend to Table I. The birds were part of the flock raised and maintained at the University of Maryland Poultry Farm. They were caponized when about 4 weeks of age and were transferred to the laboratory as young adults in the early Fall. The capons were kept, 2 to a large cage (hen turkey size), in one battery. Food and water were available at all times; light hours were held at 14/day. Three birds were allocated to each treatment and the birds were so arranged that one uninjected and one Pg-only injected capon, respectively, appeared as one of a group of 4. In this manner all capons were subjected to similar disturbance at handling. All birds were caged for 4 months prior to start of experiment with monthly recording of length and depth of comb and of body weight. These same records were continued for 2 months beyond completion of test. In all fowl, feathers of suitable regeneration were microscopically examined for structural developments indicative of abnormally elevated thyroidal activity: no such reactions were found. The birds when first placed in cages were molting heavily. Thereafter they continued the minor sporadic shedding that is a castrate characteristic. This property, more apparent in certain regions, introduces some little difficulty in evaluation of degree of experimentally provoked molt. We recognized as induced molt a distinct activation of feather papillae initiated along the known molt lines (embryonic tract axes) and from which an orderly spread took place. Relative degree of molt (+ to 4+, Table I) was then scored as the estimated respective distance of that spread.

Results. The experimental castrate molt, when it occurred, began on day 18 from day 1 of treatment or 6 days after cessation of injections. It was always of sharply limited, brief duration. Pg led to molt wherever used. FSH and LH were negative; when injected in conjunction with Pg, the effect was comparable to that of Pg alone. LTH called forth an unmistakable activation of the feather papilla in all capons although the response was more violent in one bird than in another. A repetition of the LTH injections in the same dosage but with the highly purified preparation referred to was equally positive in molt. Table I shows that the effects in the papilla with this treatment were entirely comparable to those obtained with the first treatment. Curiously, however, the purified preparation provoked a local reaction wherever used; sites of injection were more highly affected in the capon (C 63), giving the slightest response in molt.

Discussion. Our observations that prolactin will stimulate feather papilla of the male castrate fowl extends the already established activities of this pituitary hormone in birds. Prolactin was first identified and described by Riddle, Bates and Dykshorn(4,5) and sub-

† Repositol Progesterone, Pitman-Moore Co.

‡ The FSH, LH and LTH preparations were most kindly furnished by Dr. I. M. Bunding, Armour and Co., Chicago, Ill.

§ The prolactin was a gift from the Endocrinology Study Section, N.I.H.

TABLE I. Molt Response of Capon to Prolactin, Follicle Stimulating Hormone and Luteinizing Hormone Given Singly and in Combination with Progesterone and to Progesterone Given Alone. Occurrence of molt was recorded on day 18 from day 1 of treatment in the feather tracts listed.

Capon No.	Treatment	Degree of induced molt by feather tract						
							Coverts	
		Breast	Back	Saddle	Thigh	Leg	Inner	Outer
36	None	0	+	0	0	0	0	0
40	"	0	0	0	0	0	0	0
63	"	+	0	0	0	+	0	0
38	Pg	+	2+	+	0	0	0	0
61	"	+	2+	+	+	+	0	+
46	"	+	2+	+	+	+	0	+
35	FSH	+	+	0	0	0	0	0
53	"	0	0	0	0	0	0	0
54	"	0	0	0	0	0	0	0
37	FSH + Pg	+	2+	2+	+	+	+	+
55	"	+	+	+	2+	+	+	+
56	"	+	2+	2+	+	2+	0	2+
39	LTH	3+	2+	+	3+	2+	+	3+
57	"	2+	3+	2+	2+	2+	2+	3+
58	"	+	4+	4+	3+	2+	3+	3+
41	LTH + Pg	4+	4+	4+	4+	3+	4+	3+
60	"	4+	4+	4+	3+	3+	4+	3+
42	"	3+	3+	3+	3+	2+	+	3+
43	LH	0	0	0	0	0	0	0
62	"	0	0	0	0	0	0	0
51	"	0	0	0	0	0	0	0
49	LH + Pg	2+	2+	+	2+	+	+	3+
50	"	+	2+	+	2+	0	0	+
47	"	+	+	0	+	0	0	+
36	LTH*	4+	4+	4+	4+	4+	3+	4+
40	"	3+	4+	3+	3+	3+	2+	4+
63	"	+	3+	3+	+	2+	+	+

Intensity of molt was scored + - 4+. Pg was given as a single intramusc. inj. of 25 mg, Jan. 13. FSH, 10 mg/day; LH, 5 mg/day; and LTH, 5 mg/day, were inj. subcut. Jan. 13-18, and 20-25.

* These LTH injections alone were repeated, 5 mg/day, with a highly purified preparation in untreated capons, March 3-8, and 10-15. Controls were untreated.

sequently became the subject of a great number of studies. Many of these findings, such as antigonadal action of prolactin and its growth-promoting properties in certain tissues, are highly important to the present results. However, the closest parallel exists between the states leading to broodiness in pigeon, dove and fowl and the experimental stimulation to molt we have described in the last. Prolactin can cause broody behavior in the laying hen while interrupting ovulation; it was effective to a lower degree in the cock(6). Prolactin diminished testis size of the pigeon through inhibition of endogenous FSH(7) and that same bird as male castrate will secrete crop milk when treated with LTH at the appropriate stages(8). Production of broodiness in intact Ring Doves by progesterone, desoxycorticosterone acetate and testosterone propionate(9) was ascribed to liberation of prolactin from the bird's own pituitary rather than to an antigonadal action as such. We have found these same substances productive of molt in the laying hen(3) and, although ovulation was interrupted, we questioned this as the primary mover. Our present findings, that LTH actively stimulates molt in the capon whereas FSH and LH proved negative, further suggests that prolactin may be the immediate agent in activation of the feather papilla.

Experimental effectiveness does not of itself demonstrate an identical role of the respective compound in the normal but there seems little contradictory in the juxtaposition of the sources of broodiness and molt; the prolactin secreted earlier could well prepare the change of plumage which frequently follows

upon termination of care of the young. It is obvious that any episodic release is triggered to an external event and in this connection a very curious and interesting observation has been made in some ducks. The drake here assumes no responsibility for his offspring and, upon observing signs of broodiness in his mate, takes his departure. He now joins a group of his fellows and proceeds to molt into the eclipse plumage(10). The pituitary has long been implicated in this special molt(11); does not the sympathetic performance of the drake suggest that prolactin may be the specific secretion involved?

Summary. Adult capons were treated with prolactin (LTH), follicle stimulating hormone (FSH), luteinizing hormone (LH), singly or in combination with a constant progesterone (Pg) dosage. In addition, Pg was administered alone. Pg was effective in activating the castrate feather papilla, these findings confirming earlier responses obtained in both intact sexes of fowl by others. FSH and LH were both ineffective in stimulating capon feather regeneration; in combination with Pg, the reaction was similar to that toward Pg alone. LTH uniformly caused molt of the capon feathering whether given alone, in combination with Pg, or alone as a highly purified preparation. The positive performance of LTH in the male castrate feathering described here, together with the observed lack of response toward FSH and LH in this fowl, suggest that prolactin may be the immediately effective agent in stimulating the feather papilla to production of a new plumage.

1. Shaffner, C. S., *Science,* 1954, v120, 345.
2. Juhn, M., Harris, P., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 202.
3. ———, *ibid.,* 1956, v92, 709.
4. Riddle, O., Bates, R. W., Dykshorn, S. W., *Anat. Rec.,* 1932, v54, 25.
5. ———, PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 1211.
6. Riddle, O., Bates, R. W., Lahr, E. L., *Am. J. Physiol.,* 1935, v111, 352.
7. Bates, R. W., Riddle, O., Lahr, E. L., *ibid.,* 1937, v119, 610.
8. Riddle, O., Dykshorn, S. W., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 1213.
9. Riddle, O., Lahr, E. L., *Endocrinology,* 1944, v35, 255.
10. Hochbaum, H. A., *Travels and Traditions of Waterfowl,* Chap. 10, U. of Minn. Press, 1955.
11. Walton, A., *J. Exp. Biol.,* 1937, v14, 440.

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Physiological Disposition of Heparin.* (24147)

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The property of heparin as inhibitor of blood coagulation has been known for a number of years(1). Although the exact mechanism for this action is still unknown, indications are that heparin acts primarily in preventing conversion of fibrinogen to fibrin. The extreme rapidity of its action, after injection, makes it a valuable therapeutic agent when employed clinically in early cases of coronary thrombosis. On the other hand the pharmacological effect of heparin disappears comparatively rapidly, consequently provision must always be made for prolonging blood coagulation time by continued administration of an anticoagulant. The physiology and metabolism of heparin has been slightly elucidated. Its importance and potential use have never been fully appreciated, possibly because the quantity of heparin in tissues is minute and previous methods for measuring and distinguishing it from other mucopolysaccharides are very tedious and open to question. With the preparation of radioactive heparin labeled with sulfur-35(2) the possibility was opened for quantitative metabolic studies of this substance. We reported results on clear-

* This investigation was supported by Grants from U.S.P.H.S. and Atomic Energy Commission.

TABLE I. % of Administered Radioactivity after Intravenous Injection of Heparin-S^{35}.*

	Hours after inj.			
	.25	2	24	48
Blood	82	23	0	0
Liver	4.4	22.6	28	0
Lung	3.3	12.5	14	0
Spleen	0	1.2	.8	0
Kidney	0	.5	.4	0
Heart	0	.11	.1	0
Urine			24	0

* Each figure is avg of results obtained from at least 3 experiments.

ance of heparin from the bloodstream(3). The present paper reports the distribution of heparin in tissues and its excretion from urine after intravenous injection.

Methods. The radioactive heparin-S^{35} used was prepared in our laboratory as described previously(2). This material, as the sodium salt, with specific activity of 1.3 X 10^4 cpm was dissolved in physiological saline to a concentration 1 mg/ml. Dogs weighing 5.4 to 6.6 kg were injected intravenously with 2 ml of radioactive heparin solution (2.6 X 10^4 cpm)/kilo body weight. In one series of experiments, the animals were sacrificed at various time intervals (Table I) and the desired organs extirpated for radioactivity assay. The organs were homogenized and duplicate aliquots were combusted and assayed as barium sulfate. A sample of the original heparin was also combusted and plated in similar manner so that a comparison between radioactivity of tissues and of heparin could be made. In another series, the injected animals were placed in metabolism cages 4 days and urine collected daily was assayed for radioactivity. All samples were counted with a gas flow counter and sufficient counts were taken to obtain an accuracy of ± 10%. The values obtained were converted to the number of cpm at infinite thinness.

Results. Following intravenous injection with a dose of 2 mg/kilo the heparin-S^{35} is rapidly cleared from the bloodstream. After 2 hours only 23% of radioactivity can be detected (Table I). A considerable portion of radioactivity accumulates in the liver so that after 24 hours, this organ accounts for 28% of the injected material. It might be noted, however, that after 48 hours, there is no measurable amount of radioactivity in the liver or any other organ, probably due to rapid turnover of heparin in the living organism, although the possibility that there is desulfation, whereby the label is lost, cannot be completely excluded. The presence of heparinase, as reported in rabbits by Jacques(4), could not be found in our dogs. However, other modes of degradation and desulfation are quite possible. In addition to liver, an appreciable amount of injected heparin accumulates in the lungs, while only minute quantities are found in spleen, kidney, or heart. On the other hand, if one considers comparative weights of organs and amounts of radioactivity/g of tissues, there is no preferential accumulation in any tissue. Excretion of radioactivity from urine is comparatively rapid so that 45-55% of the injected dose is excreted within the first 24 hours (Fig. 1). In one experiment, urine was obtained from animal 30 minutes after injection and 2% of radioactivity had already been excreted. Over 60% is excreted after 48 hours; 75-85 and 80-90% after 72 and 96 hours, respectively.

The rapid removal of radioactivity from the bloodstream is a clear indication that disappearance of the pharmacological effect after a comparatively short interval is not due to deactivation or neutralization of the heparin but rather to actual loss of this substance from the circulatory system. In addition, results with other tissues demonstrate that heparin has a rather rapid turnover rate so that it is

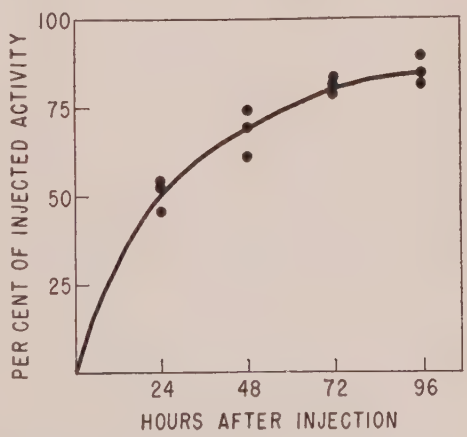

FIG. 1. Excretion of radioactivity in urine of dogs after inj. of Heparin-S^{35}. Dose was 2 mg (specific activity 2.6 × 10^4 cpm)/kg body wt.

almost completely excreted within a few days.

Summary. After intravenous administration of heparin-S^{35} to dogs, it is rapidly cleared from the bloodstream. The highest radioactivity is found in liver and lung. However, no measurable radioactivity is detected in any organ after 48 hours.

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Evidence for Conversion of Corticosterone to Aldosterone in Man.* (24148)

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Although corticosterone (compound B) might reasonably be considered a biosynthetic precursor of aldosterone (18-aldehydo-corticosterone), conversion of the former steroid to the latter has not previously been demonstrated in man. Moreover, studies using animal tissues have yielded conflicting results. Rosemberg and Dorfman(1) found only inconsequentially increased secretion of aldosterone-like material by calf adrenals perfused with corticosterone. Wettstein(2) reported actual suppression of aldosterone synthesis when beef adrenal homogenates were incubated with compound B. In contrast, upon incubating beef adrenal capsule strippings with corticosterone-4-C^{14}, Travis and Farrell recently isolated aldosterone having a molar specific activity 54% that of the labeled corticosterone(3). The present study revealed that prolonged, continuous infusion of corticosterone† to normal man simultaneously caused sharply increased urinary aldosterone and profound depression of 17-hydroxycorticosteroids. The alterations in steroid excretion were accompanied by marked shifts in urinary electrolytes, but no metabolic evidence of enhanced glucocorticoid activity.

Material and methods. Three male patients, 33 to 43 years of age, having no organic disease were studied. During a 3-day control period on hospital diet, daily sodium and potassium excretions remained essentially constant. On days 4 and 5 corticosterone was administered continuously by vein, patient A receiving 200 mg daily and patients B and C receiving 300 mg/24 hours. Daily steroid was infused in 2000 ml of 5% glucose in water, except that patient C inadvertently received his first 300 mg of compound B in 2000 ml of 0.9% NaCl. Aliquots of 24-hour collections of urine were analyzed by flame photometry for sodium and potassium, and by standard methods for glucose,‡ creatinine(4) and uric acid(5). Urinary aldosterone was assayed by the physico-chemical method of Neher and Wettstein(6), and 17-hydroxycorticosteroids by the Porter-Silber procedure(7).

Results. Qualitatively similar changes were shown by all 3 patients during and after perfusion with corticosterone (Table I). Excretion of aldosterone at least doubled during steroid administration, then fell to sub-baseline values in the recovery period before regaining normal levels. Mean daily aldosterone titers for the group were 8.6 μg in the control period, 20.0 μg at peak excretion during corticosterone infusion, and 6.1 μg on the second day of recovery. Simultaneous mineralocorticoid effects were evidenced by a sharp fall in Na/K ratio during the infusion period (from mean control value of 1.2 to minimum of 0.4), the decrease reflecting

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TABLE I. Daily Urinary Excretion of Aldosterone (μg), 17-Hydroxycorticosteroids (mg), Sodium and Potassium (Na/K) during and after Administration of Corticosterone Intravenously.

		Patient A (200)*			Patient B (300)			Patient C (300)			Mean		
Day	Period	Aldo	Na/K	17-OH	Aldo	Na/K	17-OH	Aldo	Na/K	17-OH	Aldo	Na/K	17-OH
1–3	Control	5.0	1.0	10.7	11.8	1.0	6.2	8.9	1.6	10.5	8.6	1.2	9.1
4	Corticos-terone	7.6	.6	4.2	21.3	.3	2.6	31.2	.3	3.5	20.0	.4	3.4
5	"	12.8	.9	1.4	15.6	.4	.4	15.7	1.5†	1.4	14.7	.9	1.1
6	Recovery	6.6	3.5	7.1	10.2	2.0	7.1	14.1	5.0	11.6	10.3	3.5	8.6
7	"		2.8	8.8	7.2	6.2	6.6	5.0	5.4	18.0	6.1	4.8	11.1
8	"				6.7	6.7	6.6	9.5	4.8	13.0	8.1	5.8	9.8

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marked sodium retention coupled with moderate potassium diuresis. Reciprocal and actually excessive shifts in sodium and potassium excretion, appearing immediately after stopping corticosterone and persisting throughout the recovery period, increased mean Na/K to 3.5 on the first post-infusion day. In contrast to its striking enhancement of mineralocorticoid activity, exogenous corticosterone appeared to suppress adrenal secretion of glucocorticoids, since average daily output of 17-hydroxysteroids plummeted from the control level of 9.1 mg to a minimum of 1.1 mg on the second day of infusion. However, prompt resumption of normal 17-hydroxycorticoid excretion (8.6 mg) appeared on the first day of recovery. Daily urinary glucose and uric acid/creatinine ratio showed essentially no changes from control values either during or after administration of corticosterone (data not shown).

The patterns of aldosterone excretion by both patients who received 300 mg of corticosterone daily exhibited identical features of special interest (Table I). Maximal increases of urinary aldosterone were attained on the first day of infusion (from control values of 11.8 and 8.9 to 21.3 and 31.2 μg per day, respectively). During the second day of sustained high-dosage perfusion with corticosterone, respective excretions unexpectedly fell to 15.6 and 15.7 μg. Aldosterone titers then continued to decline progressively after the infusion was stopped, and reached sub-baseline values of 7.2 and 5.0 μg on the second day of recovery. Despite seemingly premature decrease in aldosterone excretion, profound reduction of both urinary Na/K and 17-hydroxycorticoids persisted until administration of corticosterone was discontinued, whereupon they rebounded vertically within 24 hours to supra-normal and normal values, respectively.

Discussion. The present data strongly suggest that normal man is capable of oxygenating corticosterone to aldosterone. Considerable fortifying evidence is required, however, before corticosterone commands serious attention as a physiological precursor of aldosterone. It must first be shown that the material behaving like aldosterone in the Neher-Wettstein chromatographic systems is in fact aldosterone. Comparison with authentic aldosterone by infra-red spectrophotometry awaits satisfactory purification of representative eluates. Secondly, the infused corticosterone must be certified as the actual source of the accompanying increment in urinary aldosterone, in order to exclude indirect cause-and-effect relationships. The *in vitro* studies of Travis(3) suggest that direct transformation of exogenous compound B to aldosterone should be demonstrable by isolation of labeled aldosterone from urine following administration of tracer corticosterone-4-C^{14}. Lastly, with respect to the postulated oxygenation of compound B to aldosterone, present data do not elucidate the anatomical site of conversion. Although the adrenal cortex attracts primary attention, an extra-adrenal locus (*e.g.*, the liver) remains a possibility. Studies in the adrenalectomized patient are clearly necessary.

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may have directly suppressed endogenous synthesis of aldosterone. This is one interpretation of the fall in urinary aldosterone during the second day of infusion with compound B, followed by sustained depression of aldosterone output for 2 days or more after corticosterone was discontinued. An alternative explanation is that decreased endogenous aldosterone secretion merely represented a compensatory response to marked sodium retention induced by corticosterone itself. Final judgment will depend upon the pattern of aldosterone excretion during administration of corticosterone to a subject on very low sodium intake.

The essentially constant excretion rates of glucose and uric acid during and after administration of corticosterone imply that the latter was devoid of glucocorticoid activity. This negative finding was surprising in view of Conn's detailed studies showing that compound B not only exerted mild effects upon organic metabolism in normal subjects, but was able to maintain patients with Addison's disease in electrolyte, carbohydrate and nitrogen balance(8).

Finally, the present data indicate that continuous high-dosage infusion of corticosterone suppressed endogenous corticoid secretion, either by inhibiting pituitary release of corticotrophin or by blocking formation of 17-hydroxysteroids within the adrenal cortex at a biosynthetic step proximal to 21-hydroxylation of 17-hydroxyprogesterone. In any event, corticosterone was not transformed to hydrocortisone (*i.e.*, Porter-Silber chromogens), a finding which supports the thesis that existence of 11-β-hydroxylation blocks subsequent hydroxylation at the C-17 position(9).

Summary. Continuous administration by vein of 300 mg of corticosterone daily for 48 hours to 2 normal men caused sharp rise in urinary aldosterone, retention of sodium, and depression of urinary 17-hydroxycorticosteroids the first day. On the second day aldosterone values began to fall, while sodium retention persisted and 17-hydroxysteroid output was negligible. During the recovery period aldosterone excretion remained below baseline levels for 2 or 3 days, whereas both profuse sodium diuresis and normal 17-hydroxycorticoid excretion appeared promptly on the first day. Essentially similar but less intense effects were exhibited by 1 patient who received 200 mg of corticosterone daily for 2 days. The data suggest that normal man is capable of performing 18-oxygenation of corticosterone to aldosterone. Should further study establish that this conversion occurs within the adrenal cortex, the tentative thesis will be fortified that corticosterone is a physiological precursor in the biosynthesis of aldosterone.

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1. Rosemberg, E., Rosenfeld, G., Ungar, F., Dorfman, R. I., *Endocrinol.*, 1956, v58, 708.

2. Wettstein, A., Anner, G., *Experientia,* 1954, v10, 397.

3. Travis, R. H., Farrell, G. L., *Fed. Proc.,* 1958, v17, 324.

4. Bonsnes, R. W., Taussky, H. H., *J. Biol. Chem.,* 1945, v158, 581.

5. Forsham, P. H., Thorn, G. W., Prunty, F. T. G., Hills, A. G., *J. Clin. Endocrinol.,* 1948, v8, 15.

6. Neher, R., Wettstein, A., *J. Clin. Invest.,* 1956, v35, 800.

7. Silber, R. H., Porter, C. C., *J. Biol. Chem.,* 1954, v210, 923.

8. Conn, J. W., Fajans, S. S., Louis, L. H., Johnson, B., *Proc. 2nd Clin. ACTH Conference,* vI, 1951, Blakiston Co., Philadelphia, 221.

9. Hechter, O., Pincus, G., *Physiol. Revs.,* 1954, v34, 459.

Received June 2, 1958. P.S.E.B.M., 1958, v98.